Biocatalytic Methods for the Hydroxylation of Non-Activated Carbon Centres

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Finally I have to thank those closest to me - James for everything; Carolyn – the best landlady, sister and friend I could ever ask for and finally Mum and Dad for their constant support. I would not be here if it were not for you. Thank you.

Abbreviations

Ac	acetyl
ADH	alcohol dehydrogenase
APCI	atmospheric pressure chemical ionisation
ATCC	American Type Culture Collection
ax	axial
Bn	benzyl
bp	boiling point
br	broad
Cbz	benzyloxycarbonyl
d	doublet
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DCU	dicyclohexylurea
d.e.	diastereomeric excess
DEPT	distortionless enhancement through polarisation transfer
DHP	3,4-dihydro-2*H*-pyran
DMAP	4-dimethylaminopyridine
e.e.	enantiomeric excess
EI	electron impact
eq	equatorial
ES	electrospray
Et	ethyl
FAB	fast atom bombardment
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
hfc	3-(heptafluoropropylhydroxymethylene)-(+)-camphorate
HPLC	high performance liquid chromatography

m	multiplet
Me	methyl
min	minutes
mol %	molar percentage
MOM	methoxymethyl
mp	melting point
MTPA	α-methoxy-α-trifluorophenyl acetic acid
m/z	mass to charge ratio
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NCIMB	National Collection of Industrial and Marine Bacteria
NMR	nuclear magnetic resonance
nOe	nuclear Overhauser effect
op	optical purity
PCP	pentachlorophenol
Ph	phenyl
ppm	parts per million
PTSA	*p*-toluenesulfonic acid
s	singlet
spp.	species
t	triplet
THF	tetrahydrofuran
THP	tetrahydropyran

Contents

1 Introduction

1.1 Biohydroxylations

Biocatalysis is widely accepted as a viable alternative to traditional chemical methods offering good chemo-, regio and stereoselectivity. The source of this selectivity is the chiral nature of the biocatalyst that, as a protein, is made up of naturally occurring L-amino acids; the protein structure interacts with the substrate molecule, placing the substrate in a particular orientation thus conferring selectivity on the transformation.

Biocatalysis is primarily utilised in the pharmaceutical and specialised organic sector due to the need to produce compounds in homochiral form and the benign conditions in which biocatalysts operate. An increasing number of processes combine biocatalysis and chemical methods into one synthetic process, with resolution by hydrolysis being the most common.[1]

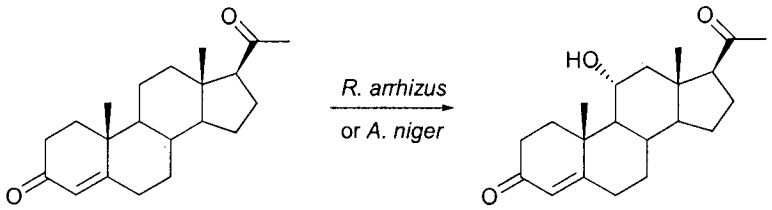
Greater understanding of enzyme function at the genetic level leading to the genomic based identification of novel catalytic activity and subsequent improvement of catalytic selectivity and efficiency through directed evolution of biocatalysts are thus likely to remain growth areas and increase the value and utility of biocatalysts.[2,3]

Of particular interest and importance are the biocatalysts involved in hydroxylation. Under the control of a biocatalyst, hydroxylations that are still chemo-, regio and stereoselective can occur at positions remote from existing functionality. Highly selective hydroxylations of this type are difficult to carry out chemically. Research for chemical methods for non-activated carbon hydroxylation have concentrated on biomimetic systems. The most comprehensive to date have been the 'Gif' systems investigated by Professor D. H. R. Barton[4] and the biomimetic systems studied by

Professor R. Breslow.[5] Although these systems go some way in terms of mimicking the selectivity of biological systems, they certainly do not provide a general method. Biocatalytic reactions therefore remain the more successful and widespread method of selective hydroxylation.

As indicated, one of the key advantages of utilising biocatalysis is the selectivity imparted on the transformation by the biocatalyst. There are numerous cases, such as the hydroxylation of camphor by *Pseudomonas putida* (discussed in section 1.2), where the biocatalyst is extremely selective in the transformation of its natural substrate. More importantly, since the exclusive use of natural substrates would distinctly limit the methodology, it is encouraging that hydroxylation of non-natural substrates can also be carried out with good selectivity.

Hydroxylations of steroids have historically been the most important use of whole cell biohydroxylation methodology. Fungi in particular have been shown to be very useful in selective steroid hydroxylation. Initially, *Rhizopus arrhizus*[6,7] and *Aspergillus niger*[8] were shown to transform progesterone **1** to 11α-hydroxyprogesterone **2** (figure 1). This discovery led to a synthesis 10-12 steps shorter than the existing chemical route in which the bioproduct was easily inverted to the required β anomer, thus allowing cost-effective production of such steroids.



1 **2**

Figure 1: Biohydroxylation of steroids

Although this initial literature is now dated, microbial transformation remains the method of choice for steroid hydroxylation. Many years of research have resulted in the knowledge that virtually any carbon centre in the steroid nucleus can now be hydroxylated selectively.[9]

Terpene hydroxylation products are of particular interest to the fragrance industry and as intermediates for the pharmaceutical industry. As for steroids, controlled chemical hydroxylation is difficult due to the large number of potential sites of hydroxylation and successful production of such compounds is mainly restricted to microbial methods.

The indisputable success of biohydroxylation methodology in naturally occurring compounds, such as steroids and terpenes, has provided the basis for recent research that has concentrated on the microbial hydroxylation of non-natural compounds.

1.2 Cytochrome P-450 monooxygenases

Monooxygenases are a class of oxidoreductases that catalyse the insertion of one atom from molecular oxygen into the substrate, while the second atom is reduced to water. An important group of monooxygenases are the cytochrome P-450 monooxygenases. These haem-dependent monooxygenases carry out the NAD(P)H dependent oxidation of a diverse range of compounds. Cytochrome P-450 monooxygenases are extremely common in nature and are found in virtually all organisms.

Substrates for cytochrome P-450 monooxygenases include hydrocarbons, fatty acids, steroids, terpenes and xenobiotic drug molecules. The enzymes perform a number of functions including aliphatic and aromatic hydroxylation, heteroatom oxidation and dealkylation and epoxidation. Perhaps most interesting is their hydroxylation of non-functionalised hydrocarbon centres as described in figure 2, since this transformation is difficult to carry out chemically.

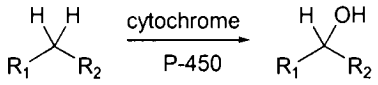


Figure 2: Hydroxylation of non-activated carbon centres by cytochrome P-450 monooxygenases

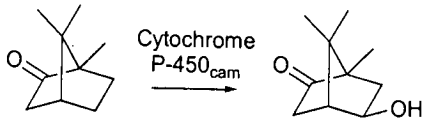
Mammalian cytochrome P-450 monooxygenases are particularly abundant in detoxification organs such as the liver, where they play an important role in the detoxification and excretion of xenobiotic molecules. They often catalyse initial hydroxylation of lipophilic compounds introducing them into breakdown pathways or making them more hydrophilic so facilitating excretion from the body. Perhaps due to this function and the need to accept a range of compounds, mammalian cytochrome P-450 monooxygenases tend to display wide substrate specificity that contrasts with their more specific prokaryotic counterparts.

Since cytochrome P-450 monooxygenases in the liver are important in drug metabolism and excretion, much of the research on the mammalian enzymes has concentrated on the identification of inhibitors that could increase the lifetime of the drug molecule in the body.[10] Unfortunately, drug molecules themselves may inhibit cytochrome P-450 monooxygenases leading to potentially harmful accumulation of other lipophilic compounds in the body.[11]

Eukaryotic cytochrome P-450 monooxygenases from fungal sources have proved very difficult to study due to instability of the protein and low expression levels and there remain few examples.[12]

Unlike their eukaryotic counterparts, prokaryotic cytochrome P-450 monooxygenases tend to display very strict substrate specificities that are generally confined to the natural substrate and its analogues. Since many monooxygenases are membrane bound, there are few examples of stable, isolated cytochrome P-450 monooxygenases, a problem that

has slowed the progress in research in human cytochrome P-450 monooxygenases. An unusual example of a stable isolated cytochrome P-450 monooxygenase is cytochrome $P\text{-}450_{cam}$ which was originally isolated from *Pseudomonas putida* and has been purified to homogeneity.[13-15] Cytochrome $P\text{-}450_{cam}$ carries out the highly regio- and stereoselective 5-*exo* hydroxylation of the monoterpene camphor **3** to **4** as shown in figure 3. This enzyme system typifies the prokaryotic cytochrome P-450 monooxygenases with substrate specificity restricted to close structural analogues of camphor such as norcamphor.



3 **4**

Figure 3: Hydroxylation of camphor by cytochrome $P\text{-}450_{cam}$

Cytochrome $P\text{-}450_{cam}$ is however a good example of the high regioselectivity possible in biocatalysis, where only one out of a possible of ten regioisomers is produced by hydroxylation. The availability of crystal structures of both free and substrate-bound protein[16-18] and the unusual stability of the protein mean that the enzyme has often been used as a model for other cytochrome P-450 monooxygenases. A recent study though has suggested that microbial systems such as cytochrome $P\text{-}450_{cam}$ cannot be used as models for mammalian metabolism.[19]

1.3 Practicalities of biohydroxylations

Although the potential advantages to using biocatalysis such as mild temperature, pH and environmentally preferred solvents are widely accepted, there are also a number of operational features which need to be addressed when using a biocatalyst. Due to

difficulties in isolating many oxidoreductases and their subsequent instability, many biohydroxylations are carried out utilising whole-cell methodology.

Aside from increased stability of the catalyst, the added advantage of whole-cell methodology is that any required co-factors and co-proteins will be provided and recycled by the organism. This is extremely important for biohydroxylations since cytochrome P-450 monooxygenases require not only a supply of oxygen but also a source of electrons, namely NADH or NADPH, and generally two co-proteins to deliver these electrons to the enzyme. Among the disadvantages are that other enzymes in the cell can cause undesired side-reactions. Additionally the larger volume of aqueous media that is required can make the product isolation laborious.

On the other hand, the use of isolated enzymes as biocatalysts can be advantageous since the work-up is likely to be simpler, often with higher concentration tolerance compared to whole cell systems and competing side reactions are much less likely. The method is however severely compromised since isolation and purification of enzymes can be very difficult, and co-proteins also need to be produced and co-factors supplied.

Although examples of isolated enzyme catalysis do exist, they are certainly not common and due to these difficulties are likely to remain so.

Whole cell biohydroxylations are widespread in the literature mainly due to the robust nature of many such biocatalysts. Because the use of such biocatalysts require little specialist knowledge or equipment, it is likely that whole cell methodologies will remain the method of choice.

1.4 *Beauveria bassiana* ATCC 7159

Beauveria bassiana ATCC 7159 is a member of a small family of fungi and is one of the most frequently used whole-cell biocatalysts. It is thought to contain a number of hydroxylase enzymes[20] and it is their function which has been most studied. Due to its widespread use as a biocatalyst, the literature is too wide ranging to carry out a comprehensive review here, but a comprehensive review of the biocatalytic reactions of *Beauveria* spp. has recently been published.[21]

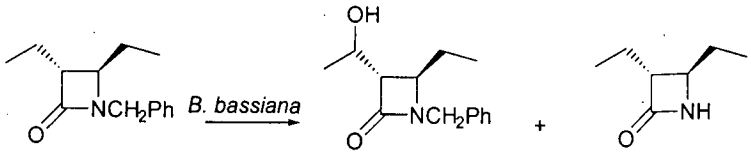
Among the biocatalytic reactions catalysed by *Beauveria bassiana* are many that are typically carried out by cytochrome P-450 monooxygenases such as aliphatic and aromatic hydroxylation; sulfoxidation and heteroatom dealkylation reactions. A number of other whole cell transformations such as double bond reduction[22] and Baeyer-Villiger oxidation[23] have also been reported.

The bulk of the literature of *Beauveria bassiana* has centred on the action of its hydroxylase activity; some key areas of which will be discussed here. It should be noted when examining the literature that the species under investigation has been redesignated twice and that *Sporotrichium sulfurescens* and *Beauveria sulfurescens* both refer to the strain under study but that the ATCC strain number is constant throughout.

1.4.1 Non-hydroxylating transformations

In the only reports of sulfoxidation by the organism, Holland and co-workers have described a combination of biocatalytic and chemical routes to reach all the stereoisomers of some amino acid sulfoxides, affording a superior route to the existing resolution-based methods.[24,25]

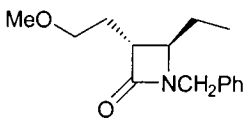
Heteroatom dealkylations by *Beauveria bassiana* also appear in the literature. β-lactam **5** not only undergoes hydroxylation of an ethyl side chain **6** but also yields a significant amount of debenzylated, non-hydroxylated product **7** (figure 4).



5 **6** (10 %) **7** (20 %)

Figure 4: Incubation of β-lactam derivative **5** with *Beauveria bassiana*

This compound was the only one in a series of five structurally related compounds, which underwent *N*-debenzylation that is thought to occur *via* benzylic hydroxylation followed by hydrolysis of the resultant aminoacetal.[26] Another compound in the series **8**, which did not show *N*-debenzylation, did undergo *O*-demethylation in a very high yield *via* hydroxylation of the methyl group to a hemiacetal followed again by hydrolysis. The difference in selectivity of hydroxylase action on these compounds indicates the large impact that seemingly very minor substrate alterations can have on the selectivity of an enzyme.



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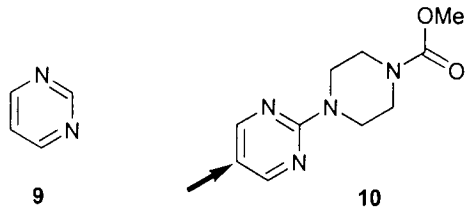
1.4.2 Aryl hydroxylation

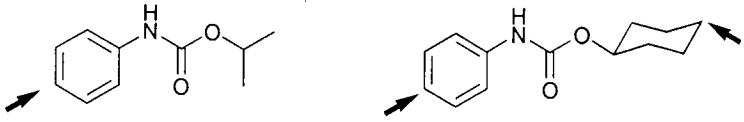
Hydroxylation of aryl carbons by *Beauveria bassiana* has been widely reported for many different compounds. One example is the hydroxylation of pyrimidine heterocycles that is seen to occur only on the aromatic ring. Successful hydroxylation was thought to be dependent on the existence of an electron rich centre in the substrate

since incubation of substrate **9** and alkyl substituted analogues was unsuccessful, whereas pyrimidine **10** was hydroxylated successfully.[27] This hydroxylase activity was seen however to be very limited when tested with substrate analogues. Other organisms such as *Rhodococcus erthyropolis* were, for these substrate types, found to be more general hydroxylation catalysts.



9 **10**

Highly regioselective *para*-hydroxylation by *Beauveria bassiana* was noted to occur in Propham **11**[28] and simple derivatives, but regioselectivity was lost on substitution with larger alkyl carbamates **12**[29] yielding mixtures of alkyl hydroxylated and aryl/alkyl dihydroxylated compounds.



11 **12**

It is clear from this example that prediction of hydroxylation selectivity is difficult, even in related compounds and that competing reactions often occur in transformations.

1.4.3 Aliphatic hydroxylation

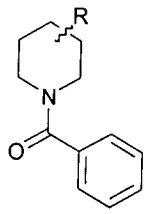
Natural substrates

Given the range of compounds that *Beauveria bassiana* hydroxylates with good selectivity, it is perhaps surprising that steroids have proved less successful as substrates for *Beauveria bassiana* compared to other biocatalysts and there are only a few examples of this activity.[30] *Beauveria bassiana* has also been utilised in the

hydroxylation of hydrocarbons and alcohols[31] and terpenes[32] but these substrate types have attracted only limited interest.

Amide and related substrates

By far the most prolific research in *Beauveria bassiana* has been in the hydroxylation of non-activated carbon centres in substrates containing a remote 'directing group'. This work was initiated by a methodical examination of hydroxylation in a long series of papers by Upjohn research group. The Upjohn group, in common with many others, have concentrated on the hydroxylation of hetero- and carbocycles often containing an electron rich substituent; typical structures being **13**[33], **14**[34] and **15**.[35]



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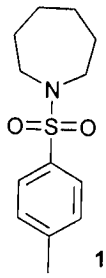

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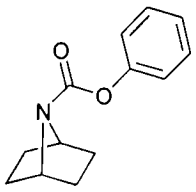

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Since the initial reports by the Upjohn research group in the late 1960s, there have been many reports of aliphatic hydroxylation by *Beauveria bassiana*, some of which have been very selective.

A study by Roberts, Willetts and co-workers demonstrates that an electron rich substituent is not a prerequisite for hydroxylation by the fungus and that regioselectivity of hydroxylation can be directed. They showed that substituting and effectively blocking the preferred site of hydroxylation can significantly alter the regioselectivity of hydroxylation of *N*-arylamines directing hydroxylation away from the aryl portion and into the aliphatic ring (figure 5).[36]

Unsubstituted *N*-arylpiperidine **16** was hydroxylated regioselectively to give the *N*-(4-hydroxyphenyl)piperidine **17** whereas for **18**, where this *para* position was substituted

and effectively blocked, hydroxylation still took place with extremely high regioselectivity but this time on the heterocycle rather than the aryl ring **19**. Suitable *para*-substituents were found to be electron rich substituents such as nitro, cyano and acetate groups, which are thought to be important for recognition by the enzyme.

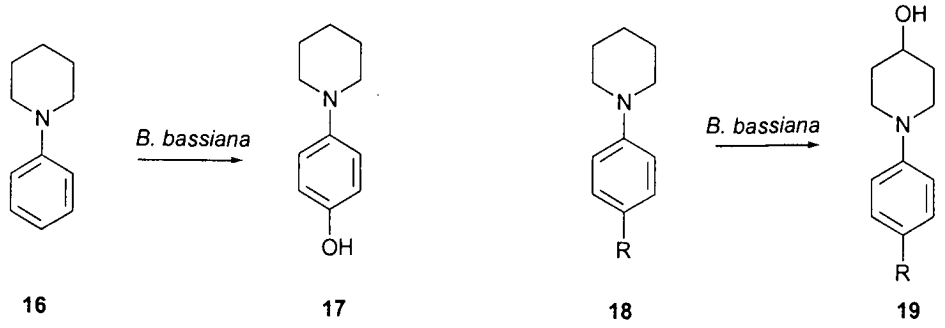


Figure 5: Regioselective hydroxylation of *N*-arylpiperidines

1.4.4 Prediction of selectivity of hydroxylation by *Beauveria bassiana*

Much of the interest in aliphatic hydroxylation by *Beauveria bassiana* has concentrated on compounds with common structural motifs. Comparison of these results has helped to produce crude models of the hydroxylase that allow prediction of site and regioselectivity of hydroxylation and for the design of new substrates. Substrates for the hydroxylases in *Beauveria bassiana* are typified by structures **13** - **15** with common features being the electron rich linkage and an aromatic side chain. The crude model shown in figure 6 was initially proposed by the Upjohn group[31] and has been widely used to predict the regioselectivity of hydroxylation in amide-based substrates.

In this model, it is proposed that cyclic system C 'fits' into the enzyme region where hydroxylation takes place and an electron rich group E such as an alcohol or carbonyl moiety interacts with the enzyme in what is referred to as the binding or anchoring region. The model also allows for some sort of lipophilic group, which may or may not be part of the cyclic system. The key restraint in the model is that there is thought to be

an optimum distance between the electron rich binding moiety and the site of hydroxylation. Although this model was initially based on results from incubation of monocyclic alcohols and was based on very few data points, for many years this has been accepted as a reasonable model for the substrate-enzyme interaction and has been used to explain the regioselectivity of a number of compounds.

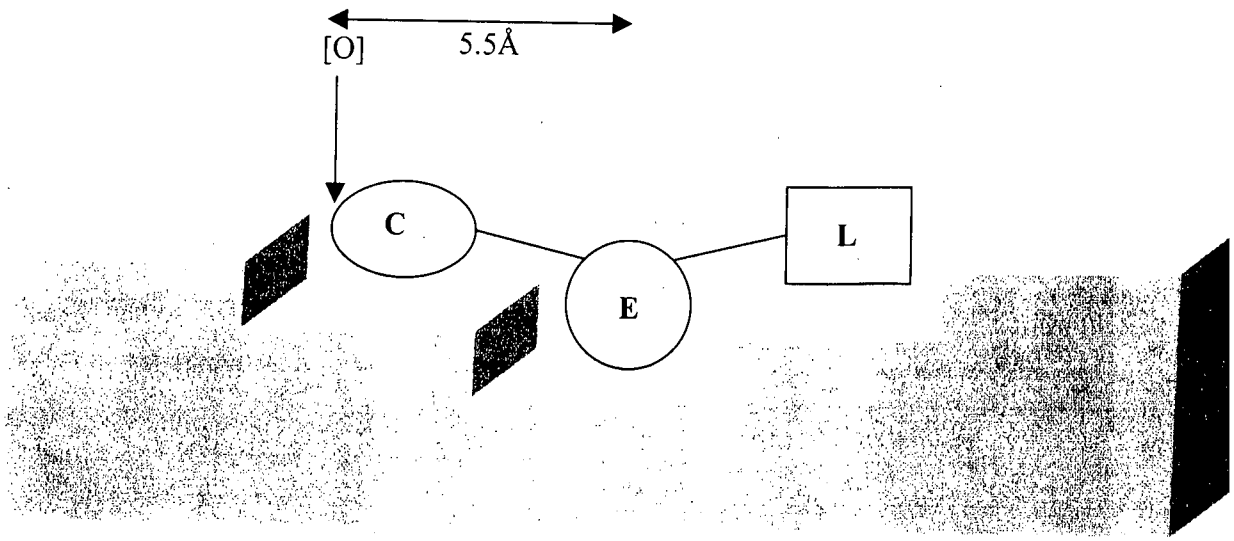


Figure 6: Crude active site model for *Beauveria bassiana*

Over the years, as more substrates were investigated, it became clear that the rigid nature of the model was not perfect and an updated version of the same model was proposed by Furstoss and co-workers (figure 7).[37] This model is much less rigid in substrate structure and allows for the electron rich substituent to be located within the cyclic system. This allows for variation in the optimum hydroxylation distance, which from experimental observations has been seen to lie between 3.5 and 7 Å from the electron rich binding group.

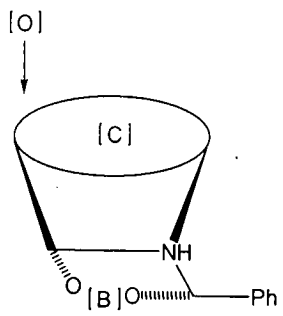


Figure 7: Improved model from Furstoss and co-workers where O is the hydroxylation site, C is a cyclic system and B is the bnding region.

This updated model does offer an explanation for the analogous hydroxylation of two substrates, one containing an exocyclic carbonyl **21** and one a cyclic carbonyl **20** (figure 8) suggesting that the location of the carbonyl was not the only determining factor in the regioselectivity.[38]

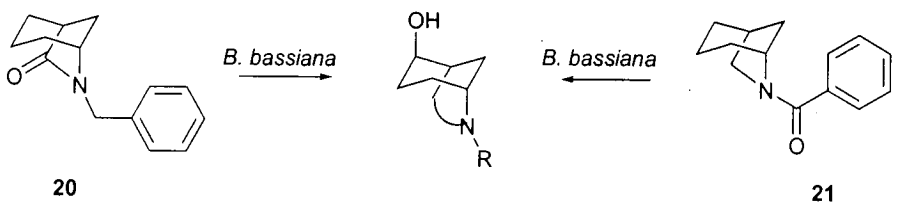
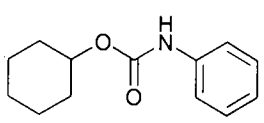


Figure 8: Analogous hydroxylation of a β-lactam and an amide

Despite many reports that the distance between an electron rich binding moiety and the site of hydroxylation seems to differ dramatically between substrates ranging from 3.5 to 7 Å, it has recently been reported by Pietz and co-workers that 5.5 Å is indeed the optimum hydroxylation distance for aliphatic hydroxylation of *N*-phenylcarbamates by *Beauveria bassiana.* They do note however that 'induced fit' of the hydrocarbon portion is also important in determining the regio- and stereoselectivity of the hydroxylation.[39] Notably their conclusions are based on small number of hydroxylations and all of the substrates contain the same aromatic side chain (**22** –**24**) perhaps explaining the

acceptability of the simplest active site model. It is unclear whether these results are broadly applicable.



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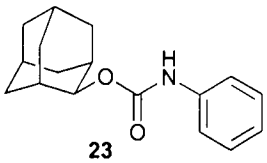

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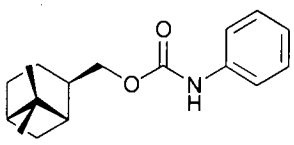

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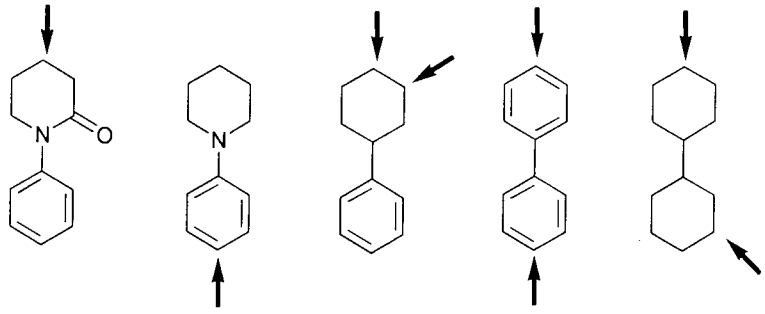
Thus far, investigations and active site models have, in the main, assumed that one hydroxylase is responsible for all of those hydroxylations studied. Given the diversity of substrate structures tolerated, whilst retaining good if differing regioselectivity, it seems unlikely that a single enzyme carries out all the transformations. Researchers had largely ignored this point until a recent examination by Holland and co-workers in which they systematically altered substrates by stepwise atom or functional group replacements and investigated the effect on the regioselectivity of transformation.[20] A typical set of substrates are shown in figure 9 and their hydroxylation sites indicated.



25 26 27 28 29

Figure 9: Series of substrates used in investigation of the hydroxylases in *Beauveria bassiana*

Holland suggests that this particular series of substrates show that the hydroxylation of these compounds is not dependent on the amide since compounds **27** – **29** are hydroxylated in similar regions to that in amide **25**. *para*-Hydroxylation of amine **26** is accounted for by the activation of this position to electrophilic attack which is perhaps a surprising explanation since one of the advantages of biocatalysis is that it is not usually dependent on activity, since non-activated groups can be transformed in the presence of other more chemically activated groups. A number of groups of compounds were similarly investigated with the results suggesting that, in some cases, regioselective hydroxylation did not seem to be dependent on an electron rich directing group.

Inhibition studies were also carried out using a range of known cytochrome P-450 monooxygenase inhibitors, which in some cases where competing hydroxylation reactions were taking place, produced a product array that was dependent on the inhibitor concentration. This combined evidence led Holland to propose that as many as four separate hydroxylases may exist in *Beauveria bassiana*. These may be responsible for arene hydroxylation; benzylic hydroxylation; non-activated aliphatic hydroxylation and the much reported hydroxylation of substrates with remote electron-rich directing groups.

This study does seem to offer a more satisfactory explanation for the selectivity of hydroxylation in such a wide range of substrates and could offer a reason for the appearance of more than one 'type' of hydroxylation in particular substrates.

1.4.5 Enantioselectivity of hydroxylation

Although in most cases, hydroxylation by *Beauveria bassiana* is regioselective, it has been noted that enantioselectivity is not always high. One notable example is the hydroxylation of rigid structures **30** and **32** (figures 10 and 11).[40]

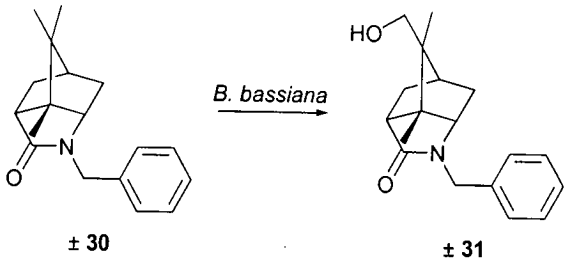


Figure 10: Regioselective hydroxylation by *Beauveria bassiana*

Lactam **30** is not hydroxylated enantioselectively but small changes in the structure changing the β-lactam into a benzoylamine **32** confer a large degree of enantioselectivity onto the hydroxylation (figure 11). From hydroxylation of optically pure starting material, it would seem that each of the enantiomers is hydroxylated on a different carbon atom such that the absolute configuration of the substrate may direct the regioselectivity.

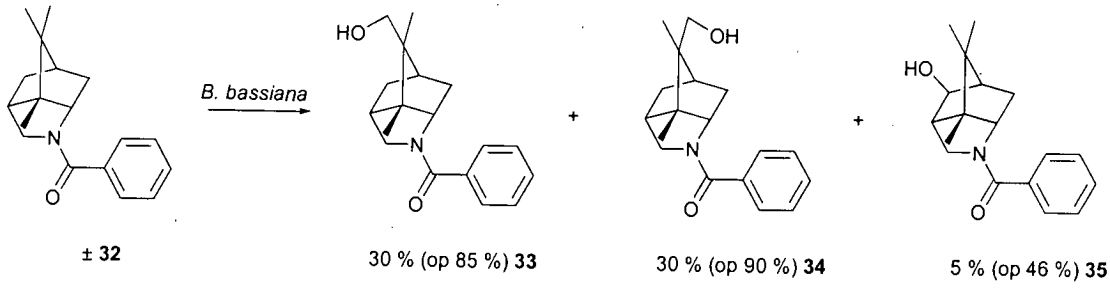
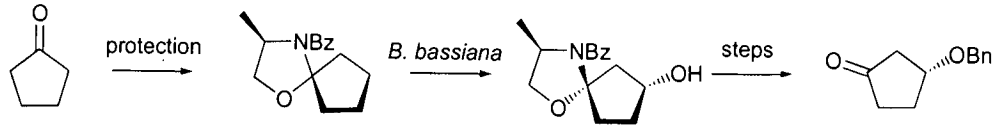


Figure 11: Enantioselective hydroxylation by *Beauveria bassiana*

In some cases, the substrate, such as simple carbocyclic ketones and carboxylic acids, may not be tolerated by the organism or may be too volatile, poorly soluble and difficult to detect. Work is currently underway in the group of Griengl to develop a general method for the successful hydroxylation of such compounds.[41] In practice, a 'docking/protecting' group (similar to a protecting group for synthetic chemistry) containing functionality, which influences substrate acceptance and hydroxylation, is

introduced prior to and removed after transformation. This approach has been extremely successful in some cases; the example shown in figure 12 is the biotransformation of cyclopentanone **36**, which is not transformed by *Beauveria bassiana* in its natural state.



36 **37** **38** **39** 84 % ee

Figure 12: Use of a 'docking/protecting' group in biotransformation

Although this method is primarily aimed at increasing the types of substrates accepted by the enzyme, the example shown uses a chiral docking/protecting group which has the further advantage that it can improve the optical purity of the product, acting much like a chiral auxiliary.

Although these and other cases do show that stereoselective hydroxylation is possible with *Beauveria bassiana*, much work is needed to ascertain the requirements for stereoselectivity.

As can be seen from this brief review of the literature surrounding *Beauveria bassiana*, although many substrates have been studied and many selective transformations identified, our understanding of the factors involved in this selectivity is limited. Overall, *Beauveria bassiana* has shown itself to be a very versatile selective whole cell biocatalyst.

1.5 Genus *Rhodococcus*

Actinomycetes are widely distributed in both soil and aquatic environments and have great metabolic diversity. The genus *Rhodococcus* is classified as a group of gram-

positive bacteria belonging to the family Nocardioform Actinomycetes. Actinomycetes have been used for industrial processes over many years and can metabolise a wide range of compounds including simple hydrocarbons, aromatic compounds and steroids, a characteristic that has increased the awareness of this family as potential biocatalysts. In common with many organisms, the first step of metabolism or transformation of many *Rhodococcus* strains is oxidation.

1.5.1 Degradation of aromatics

The degradation of aromatic compounds by *Rhodococcus* species has been well documented. Styrene, an important industrial chemical, has been shown to be metabolised by *Rhodococcus rhodochrous* NCIMB 13259,[42] an organism that can also oxidise a range of aromatic substrates including toluene.

Rhodococcus chlorophenolicus PCP-1 was isolated from a pentachlorophenol (PCP) enriched culture inoculated from lake sediment and degrades pentachlorophenol and other polychlorinated phenols.[43] Pentachlorophenol is widely used as a wood preservative and although it is known that it is biodegradable, PCP contamination of soil and groundwater is a known environmental problem.

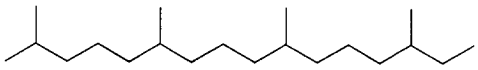
Rhodococcus spp. are generally persistent in the environment and apparently lack catabolite repression, making them potential bioremediation catalysts. This use has been suggested for both styrene and pentachlorophenol utilising strains and indeed the pentachlorophenol utilising organism has already been shown to be successful in bioremediation tests.[44,45]

Rhodococcus sp. strain C125 (*Corynebacterium* strain C125) has been shown to grow well on a range of aromatic compounds such as *o*-xylene, ethylbenzene and tetralin and contains an NAD(P)H-dependent dioxygenase activity.[46]

The *Rhodococcus* spp. demonstrate large substrate diversity even within aromatic compounds with phenols, halogenated phenols and other substituted aromatics tolerated.

1.5.2 Degradation of hydrocarbons

There are numerous reports of *Rhodococcus* species which are able to transform hydrocarbons, primarily through initial terminal hydroxylation; including substrates ranging from propane (*Rhodococcus rhodochrous* ATCC 21198)[47] and octane (*Rhodococcus rhodochrous* NCIMB 9703)[48] to branched long chain hydrocarbons such as phytane **40**.[49]



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1.5.3 Use of *Rhodococcus* spp. in biocatalysis

Due to their wide metabolic diversity, *Rhodococcus* spp. have been investigated as biocatalysts for many years. Reports of the use of *Rhodococcus* sp. as asymmetric catalysts include their use in the preparation of enantiopure epoxides and as microbial epoxide hydrolases.[50]

The biggest application of *Rhodococcus* spp. is as biocatalysts for nitrile hydrolysis. *Rhodococcus* spp. have been shown to participate in two pathways in the conversion of nitriles to carboxylic acids; either by direct conversion using a nitrilase enzyme or *via* an amide intermediate by the action of two enzymes (figure 13).

The interest in these transformations results from the value of the products and the harsh conditions (strong acid/base under reflux) required for chemical transformation. The chemical transformation also produces by-products that are difficult to remove, whereas the biocatalytic route shows high specificity. Bioprocessing systems used in industry employ *Rhodococcus* strains for the production of acrylamide[51] in one of the largest tonnage biocatalytic processes in operation.

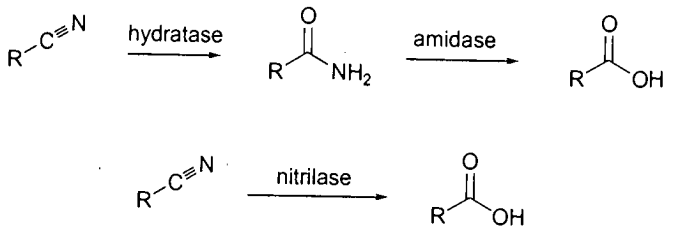


Figure 13: Biocatalytic nitrile hydrolysis

In addition to nitrile hydrolysis being chemoselective, it has been shown that hydrolysis of nitriles[52] and aminonitriles **41**[53] can also be enantioselective (figure 14). Experiments using the amide intermediate showed that in this case the nitrile hydratase activity is not enantioselective whereas the amidase activity is.

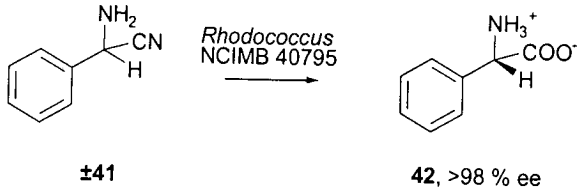


Figure 14: Enantioselective hydrolysis of aminonitriles

In a related hydrolysis reaction and in combination with another biocatalyst, a key problem in the synthetic route to carbovir **43** was overcome using *Rhodococcus equi* as a biocatalyst, allowing both enantiomers to be prepared for biological testing (figure 15).[54,55]

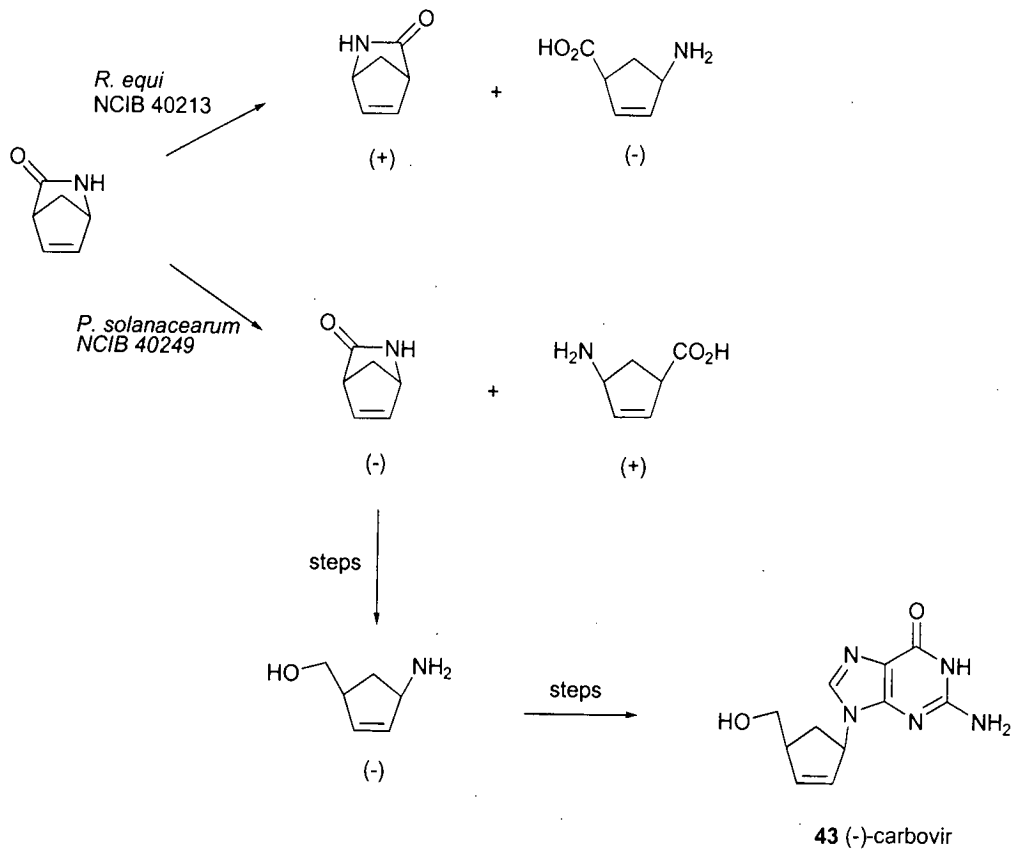
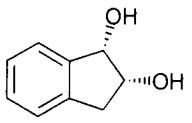


Figure 15: Preparation of carbocyclic nucleoside carbovir **43**

cis-(1S, 2R)-indandiol **44** and *trans*-(1R, 2R)-indandiol **45** are both potential precursors to (-)-*cis*-(1R, 2R)-1-aminoindan-2-ol which is a key chiral synthon for Crixivan® (indinavir) **46**, an orally active HIV protease inhibitor.

Two *Rhodococcus* species have recently been shown to have biocatalytic activity for the conversion of indene to the *cis*- and *trans*-indandiols[56] and it is hoped that this discovery could be used to provide large amounts of the enantiomerically pure intermediate and thus improve the current chemical synthesis of indinavir **46**.



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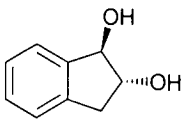

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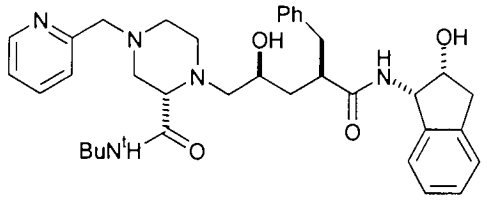


46

These last two examples show the potential utility of microbial oxidation in the synthesis of enantiopure pharmaceutically important molecules.

1.5.4 Cytochrome P-450 monooxygenases in *Rhodococcus* species

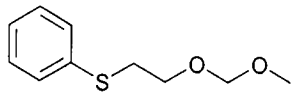
It is known that many species of the actinomycetes contain at least one cytochrome P-450 monooxygenase.[57] Investigation of such systems as biocatalysts has concentrated on the *Streptomyces* genus, although it seems likely that other species of Actinomycetes such as *Rhodococcus*, *Corynebacterium* and *Nocardia* also have potential as cytochrome P-450 mediated hydroxylation catalysts. It is interesting to note that, in a 40 page review of the biotransformations catalysed by the *Rhodococcus* spp. published in 1994,[124] the actions of cytochrome P-450 monooxygenases are not mentioned, despite their being responsible for initiating the metabolism of alkanes by hydroxylation.

Cytochrome P-450 monooxygenases have been proposed to be involved in epoxide formation by *Streptomyces flavovirens*[58] and *O*-demethylations in *Streptomyces setonii*.[59] There are also a number of cytochrome P-450 monooxygenases of actinomycete source, which have been isolated and characterised such as $P\text{-}450_{soy}$ from *Streptomyces griseus* ATCC 13273.[60] Cytochrome $P\text{-}450_{soy}$ is induced by growth on soybean flour enriched media and is interesting since it has a broad substrate specificity more typical of mammalian cytochrome P-450 monooxygenases than those of prokaryotic origin.

Although a number of *Rhodococcus* strains have been reported for their hydroxylating abilities, some of these have been shown to have non-P-450 dependent hydroxylating ability. An example of this is *Rhodococcus erythropolis*,[27] where a molybdoenzyme is thought to be responsible for hydroxylation of pyrimidine heterocycles as determined through inhibition studies using known inhibitors. There are however increasing numbers of cytochrome P-450 monooxygenases implicated in hydroxylation by *Rhodococcus* spp.

A single cytochrome P-450 monooxygenase system has recently been implicated in the degradation of herbicides such as atrazine by *Rhodococcus* sp. strain NI86/21 and has been suggested as a good target for bioremediation.[61,62]

Rhodococcus equi IFO 3730 has been shown to carry out the asymmetric sulfoxidation of 2-alkoxyethylsulfides such as **47.** In the case shown the MOM group was then cleaved to give optically pure 2-hydroxyethyl sulfoxides that are potentially useful synthetic intermediates.[63] Such sulfoxidation could be carried out by monooxygenase enzymes including cytochrome P-450 monooxygenases.



47

As noted for *Beauveria bassiana*, *Rhodococcus* spp. have also been shown to selectively hydroxylate steroids. Resting cells of *Rhodococcus* sp. IOC-77 have been shown to carry out the 9α-hydroxylation of steroids, such as 4-androstene-3,17-dione **48** *via* a hydroxylation, which may be cytochrome P-450 catalysed (figure 16).[64]

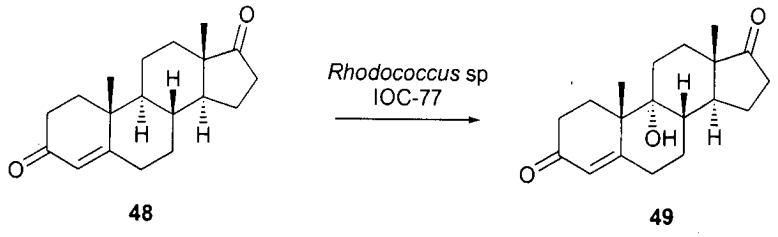


Figure 16: *Rhodococcus* catalysed hydroxylation of steroids

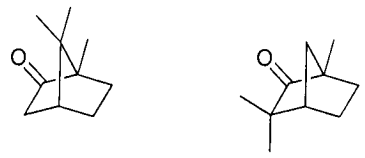
1.5.5 *Rhodococcus rhodochrous* NCIMB 9703

It has been reported that *Rhodococcus rhodochrous* ATCC 19067 (NCIMB 9703) (previously classified as *Corynebacterium* sp. strain 7E1C) grows on *n*-octane inducing a cytochrome P-450 which catalyses the NADH-dependent terminal hydroxylation of *n*-octane.[48,65] This soil bacterium was originally isolated as a propane utiliser and was reported to carry out the diterminal oxidation of long chain (C10 – C14) alkanes. It was found that growth on *n*-octane as the sole carbon and energy source induced a cytochrome P-450 system. Cell free extracts were shown to transform octane to 1-octanol and octanoic acid requiring NADH and oxygen. The hydroxylation system was separated into two fractions, one containing cytochrome P-450 and one containing flavoprotein, both of which were necessary for activity.[48,65]

Despite the ongoing interest in the *omega* hydroxylase from *Pseudomonas oleovorans,* which was discovered around this time and the ongoing interest in cytochrome P-450 systems, to my knowledge no further investigation of this organism has been undertaken.

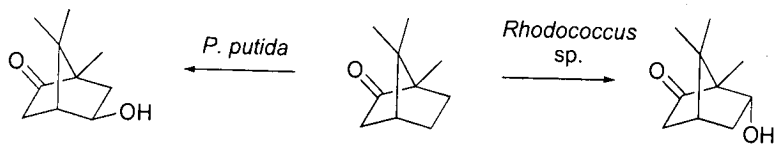
1.5.6 *Rhodococcus* sp. NCIMB 9784

Rhodococcus sp. NCIMB 9784 has been reported to perform regioselective hydroxylation of camphor **3** to produce 6-hydroxycamphor.[66] This strain was also reported to catalyse the Baeyer-Villiger biooxidation of fenchone **50**.[67] This result in itself was interesting and suggested a high degree of selectivity for the enzymes present, since camphor and fenchone have great structural similarity and yet are degraded by different initial oxidative attack.



3 **50**

Although hydroxylation of camphor by this organism was seen to be highly selective, further investigation of this organism was limited and research on camphor hydroxylation concentrated on cytochrome P-450$_{cam}$ from *Pseudomonas putida* (section 1.3). Recent work within our laboratories[68] has isolated the hydroxylase and confirmed its classification as a cytochrome P-450. Through NMR studies, the site of hydroxylation has been confirmed as the 6-*endo* position. This highly selective cytochrome P-450 dependent hydroxylation is very interesting, due to its contrasting regioselectivity with the equally selective cytochrome P-450$_{cam}$ (figure 17).



4 **3** **51**

Figure 17: Complementary hydroxylation of camphor by two biocatalysts

This complementary regioselectivity makes the camphor utilising cytochrome P-450 in *Rhodococcus* sp., an interesting target in the search for more information regarding the origin of selectivity of transformation by cytochrome P-450 monooxygenases.

Another cytochrome P-450 of Actinomycete source, cytochrome P-450_{soy} from *Streptomyces griseus* was also shown to catalyse the hydroxylation of camphor. It is noted that, in this case, the regioselectivity is much lower with four products isolated.[69] This lower regioselectivity of hydroxylation is similar to the regioselectivity of hydroxylation of P-450_{LM-2} from rabbit liver where 5-*exo*, 3-*endo*, 5-*endo* and other minor hydroxycamphor products were produced, showing an example of the similarity of eukaryotic cytochrome P-450_{soy} to its mammalian counterparts.[70]

Perhaps surprisingly, with the implication of cytochrome P-450 monooxygenases in hydroxylations by *Rhodococcus* sp., very few have been isolated and identified. One example is the identification of two cytochrome P-450 monooxygenases in *Rhodococcus rhodochrous* strain 116 which degrade aromatic ethers. These P-450's are induced by 2-ethoxyphenol and 4-methoxybenzoate respectively and the compounds inducing the expression of each P-450 binds specifically to that enzyme, suggesting a narrow substrate specificity.[71] Cytochrome P-450_{RR1} (induced by and binder of 2-ethoxyphenol) has since been purified to apparent homogeneity and fully characterised as a cytochrome P-450.[72]

1.6 Summary

For this brief review of some of the key literature, it should be clear to the reader that biohydroxylations are an important class of biocatalytic reaction and are likely to remain so for the foreseeable future. The key to this importance is the high selectivity that the enzyme catalyst imparts to the transformation and that there is, to date, no easily or widely acceptable chemical alternative.

One of the key questions in the field is the prediction of the site and selectivity of hydroxylation. This issue is being addressed particularly with the *Beauveria bassiana* hydroxylase system(s) based on substrate studies, with some success; further success is likely to depend on the isolation of the enzymes involved.

Although attempts have been made to utilise microbial hydroxylation systems as models for mammalian metabolism, it has been shown that while there are often commonalities between the microbial and mammalian systems, they have been found to be unreliable and at times can be misleading.[19]

The most important application of biocatalysis in pharmaceutical research is the use of these organisms to overcome synthetic problems and allow the production of selectively hydroxylated drug molecules. Such hydroxylated compounds are extremely important for use in identifying mammalian metabolites and for subsequent testing as a metabolite. Biocatalysis is also extremely important in the production of key chiral intermediates for synthesis.

1.7 Aims

The aims of this project were two fold. Firstly we were interested in carrying out a systematic study into the hydroxylation of a series of structurally related compounds by the well-studied *Beauveria bassiana* to investigate the impact of structure modifications on the regioselectivity of hydroxylation.

Secondly we wanted to investigate the hitherto undetermined cytochrome P-450 dependent hydroxylation potential of a number of Actinomycetes belonging to the *Rhodococcus* genus. Furthermore we wanted to investigate the scope and selectivity of any activities identified.

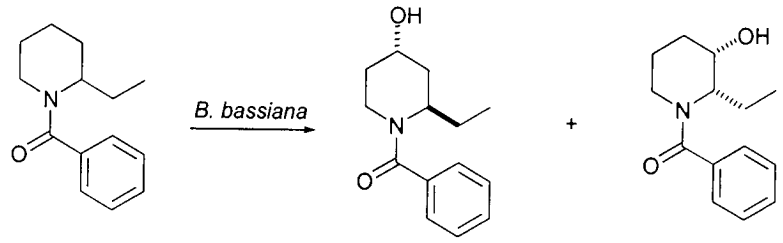
Combination of this re-investigation of a previously utilised biocatalyst with the investigation of new biocatalytic activities was hoped to yield more information regarding cytochrome P-450 selectivity and perhaps identify routes to useful chiral intermediates for use in further synthesis.

2 Biotransformations using Beauveria bassiana ATCC 7159[73]

2.1 Aims

Beauveria bassiana ATCC 7159, as discussed in the introduction, is used widely as a whole cell biocatalyst for hydroxylation. Attempts to model the substrate/active site of the hydroxylase(s) have allowed prediction of the regioselectivity of hydroxylation with some success. As indicated in the discussion of the active site models, the important factor in determining the site of hydroxylation has always been assumed to be the relative position of the electron rich moiety. Although apparently not necessary for regioselective hydroxylation, the presence of an aromatic moiety in the substrate molecule is noted in many good substrates for the hydroxylase(s). As an extension of the active site models, we were interested in the importance of this aromatic side chain, in particular on the regioselectivity of hydroxylation.

An appraisal of the literature showed that *N*-benzoylpiperidines had been discovered in the late 1960s to be hydroxylated with some regioselectivity to yield, in some cases, optically active hydroxylated piperidine derivatives (figure 18).[33]



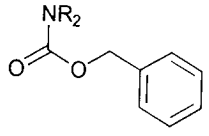
52 53 54

Figure 18: Typical transformation yielding hydroxypiperidine derivatives

It has been noted previously that seemingly minor changes in substrate structure can have a significant effect on the selectivity of hydroxylation. We envisaged that the importance of the aromatic group could be investigated by the examination of analogous compounds to those previously studied retaining the *N*-heterocycle but incorporating a different aromatic side chain.

Another important factor in our investigations was the utility of the hydroxylated products, since we wanted not only to gain further information on the structural requirements required for selectivity, but to investigate the potential for this biocatalytic route as a means of accessing selectively hydroxylated compounds. Important to this utility is the ease of removal of the aromatic side chain. Although the *N*-benzoylpiperidine transformations could be considered a potential route to hydroxylated piperidines, subsequent removal of the *N*-benzoyl group is not trivial.

N-benzyloxycarbonyl (Cbz) would appear to be a potentially more useful aromatic side chain. The group has the typical protecting group properties of ease of introduction and removal: the group is introduced *via* reaction of the free amine with benzylchloroformate and cleaved by catalytic hydrogenation or acidolysis by *O*-dealkylation, followed by loss of carbon dioxide. These methods of removal would be mild enough to leave the hydroxylated heterocycle unaffected. Most importantly for our studies, the group retains the electron rich carbonyl group, thought to be necessary for selectivity, and an aromatic group. The aromatic 'side chain' **55** is also longer than the benzoyl group **56** but the electron rich carbonyl is still directly attached to the nitrogen allowing direct comparison of the substrates (figure 19).



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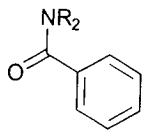


56

Figure 19: Comparison of aromatic side-chains studied

The third point under investigation is the level of substitution around the heterocycle. In Johnson's work, a number of alkyl substituents around the heterocycle were tolerated; indeed some of these alkyl side chains were actually hydroxylated. We wanted to investigate the incorporation of such substituents and their effect on the selectivity of hydroxylation.

In summary, our aims were three-fold; namely the investigation of the effect of a longer aromatic side chain with different properties to that previously investigated, the effect of introducing alkyl substituents around the heterocycle and the use of an easily removed protecting group to direct hydroxylation.

A range of *N*-Cbz protected alkyl piperidines were prepared from the corresponding commercially available alkyl piperidines by reaction with benzylchloroformate.[74]

2.2 Methods

In common with the literature, these transformations were carried out using growing cells. Since the hydroxylases in *B. bassiana* are thought to be constitutively expressed, growth was on glucose/corn steep solids media with no attempted induction. The substrate was added as a concentrated solution in ethanol to a final concentration of 0.1 mg/ml. This low substrate tolerance is often regarded as one of the main disadvantages of whole-cell methodology as, in this case, conversion of 250 mg required extraction of 2.5 L of aqueous phase. This procedure was not optimised although it was based heavily on literature methods.[34,75]

2.3 Results

Since the initial part of this work has been reported previously,[74] little detail will be given here, but the results are included to allow a complete comparison of results. The results of the biohydroxylations are summarised in table 2 (page 44) along with a comparison with results obtained using *N*-benzoyl analogues.[33] NMR experiments to ascertain the relative stereochemistry have not been reported previously.

***Incubation of N-Cbz-piperidine 57*[74]**

The unsubstituted Cbz-protected piperidine **57** is hydroxylated almost exclusively at the 4-position (**58**, 33 % yield) (figure 20). The structure was easily assigned from inspection of the ^{1}H and ^{13}C NMR spectra that showed the presence of the characteristic C*H*OH moiety and clearly showed no loss of symmetry in the hydroxylated product.

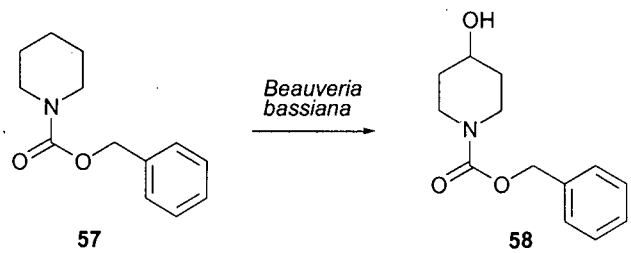
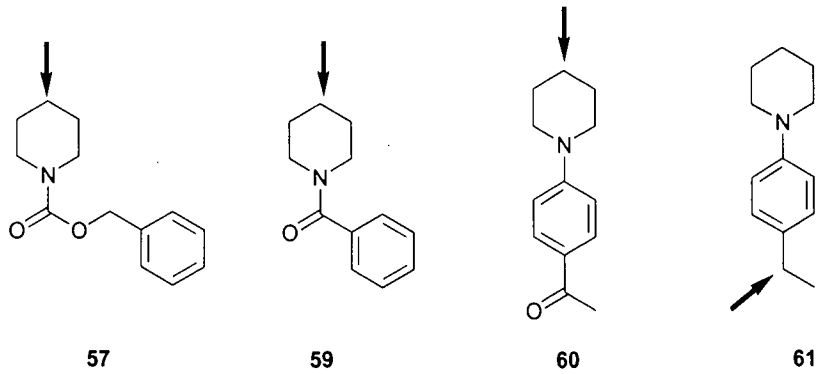


Figure 20: Transformation of alkylpiperidine **57** by *Beauveria bassiana*

The regioselectivity noted was in common with results obtained with *N*-benzoyl **59**[33] and *N*-phenyl[36] derivatives **60** and **61**. The regioselectivity in these cases is interesting because the substrates are all hydroxylated selectively in the same position, despite the common structural features, the carbonyl group and the phenyl ring, being located in different positions in the molecules (figure 21).



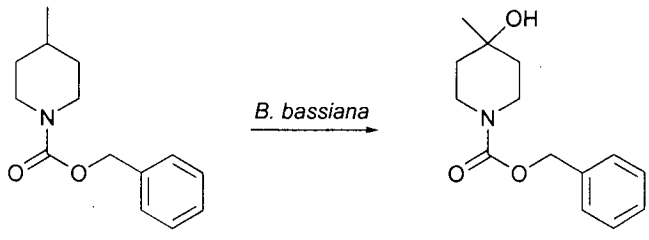
57 59 60 61

Figure 21: Known substrates for *B. bassiana* with site of hydroxylation indicated

Amides **57** and **59** have the carbonyl substituent between heterocycle and aromatic the same distance from the hydroxylation site. Amine **60** is also hydroxylated with the same regioselectivity although the carbonyl group is in this case significantly further removed from the site of hydroxylation. This electron rich group appears to be required, since regioselectivity for hydroxylation of the heterocycle is not retained in amine **61**.

Incubation of N-Cbz-4-methylpiperidine 62[74]

This high selectivity was completely conserved when the 4-methylpiperidine derivative **62** was the substrate, with hydroxylation occurring at the tertiary carbon (**63**, 45 % yield) (figure 22).

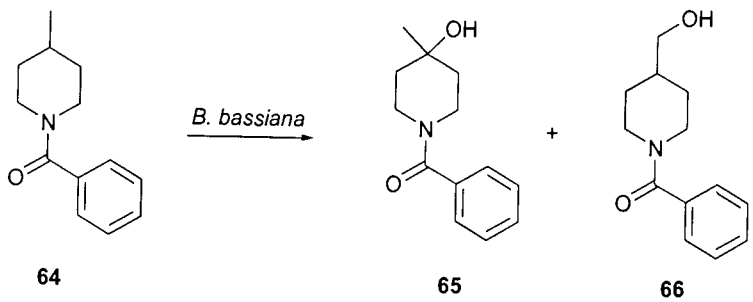


62 63

Figure 22: Biohydroxylation of substituted piperidine derivative

Although the mechanism of hydroxylation by enzymes is currently unproven, some proposals have suggested that a radical mechanism is involved in a multistage process.[76] Indeed an investigation by Fourneron has implicated the involvement of a carbon radical intermediate in biohydroxylations by *B. bassiana*.[77] In light of this evidence, it is tempting to explain the regioselectivity of hydroxylation of the 4-methyl substituted piperidine **62** in terms of the stability of the tertiary radical intermediate.

It is interesting to note that biohydroxylation of the *N*-benzoyl derivative **64**[33] resulted in a mixture of the 4-hydroxy compound **65** and the 4-hydroxymethyl compound **66** as a result of competing hydroxylation of the alkyl side chain (figure 23). In this case, the insertion of a methyleneoxy unit between the aryl group and the electron rich amide appears to have significantly improved the regioselectivity. In the case of the analogous *N*-(4-acetylphenyl)-4-methylpiperidine, substitution on the heterocycle and retention of the aryl moiety resulted in only the 4-hydroxy-4-methyl compound in common with our observations.[36]



64 **65** **66**

Figure 23: Typical transformation of substituted *N*-benzoylpiperidines

Incubation of N-Cbz-3-methylpiperidine 67

In a departure from the high regioselectivity observed for the previous reactions, three products were isolated from the incubation of *N*-carboxybenzyl-3-methyl piperidine **67** (figure 24).

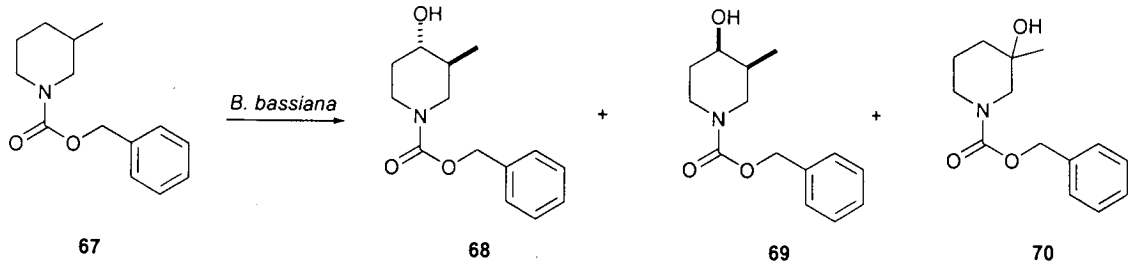


Figure 24: Mixture of products produced by biohydroxylation of **67**

The first was identified as a 4-hydroxylated product **68** by NMR spectroscopy. Coupling constants for the proton attached to the hydroxylated carbon (C*H*OH signal) also allowed determination of the relative stereochemistry of the hydroxy and alkyl substituents. The C*H*OH signal showed two large and one small couplings (*J* 3, 12 and 14 Hz) which were attributed to two axial-axial and one axial-equatorial coupling as shown in figure 25. This assignment of the coupling pattern suggests that the hydroxyl group is introduced equatorially and that the methyl group also adopts an equatorial position.

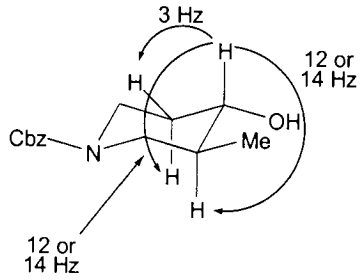


Figure 25: Coupling constants used in assigning hydroxylated product **68**

The remaining two products were found to be very difficult to separate either by flash chromatography or preparative HPLC. The mixture was therefore subjected to acetylation conditions under which only one of the two products was acetylated (as indicated by NMR spectroscopy and mass spectrometry) yielding a mixture of *O*-acetylated **69** and **70** which were then easily separated by preparative HPLC.

The *O*-acetylation of **69** to yield **69a** was confirmed by NMR spectroscopy, which demonstrated that it was an *O*-acetylated stereoisomer of alcohol **68**. In the case of **69a**, the hydroxyl group was deduced to be introduced axially since the adjacent proton signal showed only three small couplings (*J* 3, 5 and 5 Hz) and therefore had no axial-axial interactions (figure 26). It was not possible to ascertain the relative position of the methyl group from simple decoupling experiments, since the C3-H signal is not easily identifiable. It is thought however that the methyl substituent is likely to lie in an equatorial position since a 3,4-diaxial conformation would be disfavoured and thus prone to ring-flip to give the 3,4-diequatorial conformation.

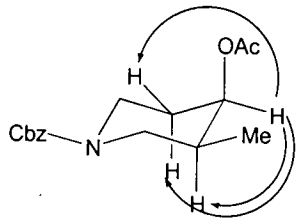


Figure 26: Couplings observed for C*H*OAc signal in **69a**

Product **70** was found to be unaltered by the acetylation conditions. Mass spectrometry (atmospheric pressure chemical ionisation) showed an $(M + 1)^+$ peak at 250.1, confirming monohydroxylation. Since the characteristic C*H*OH signal was missing in the NMR spectrum, alcohol **70** was assigned as the 3-methyl-3-hydroxy derivative.

This example shows that ease of radical formation is certainly not always the single controlling factor in hydroxylation regioselectivity. In this case, alignment of the piperidine ring in the enzyme active site is a more significant factor.

This transformation was reported previously[74] although the products were not separated and characterised. Biooxidation of the corresponding compound, *N*-benzoyl-3-methylpiperidine, produced a mixture of 3- and 4-hydroxy products giving a comparable product array.[33]

Incubation of N-Cbz-2-methylpiperidine 71[74*]

Incubation of the fungus with *N*-Cbz-2-methylpiperidine **71** yielded approximately equal amounts of two products **72** and **73** (figure 27).

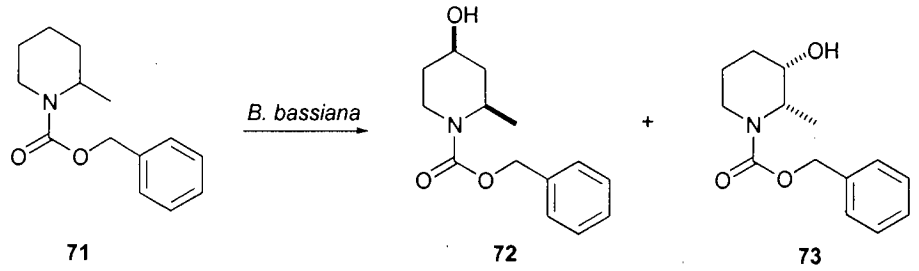


Figure 27: Biotransformation of 2-methylpiperidine derivative **71**

Alcohol **72** was found, by NMR spectroscopy, to be hydroxylated in the 4-position because the characteristic C*H*OH signal shows the maximum four couplings. All the coupling constants were shown to be small (*J* 3 Hz), indicating an axial positioning for the introduced hydroxyl group (figure 28). NOE experiments showed no enhancements providing evidence as to the relative stereochemistry of the methyl and hydroxyl substituents. It is well documented however, that for alkylpiperidine amides, 2-alkyl substituents favour axial rather than equatorial positioning[78,79] due to severe $A^{(1,3)}$ interaction in the equatorial conformation. It was thus assumed that alcohol **72** has the 2,4-diaxial arrangement.

* Biohydroxylation experiment carried out by Dr. Gideon Grogan; product characterisation carried out by the author.

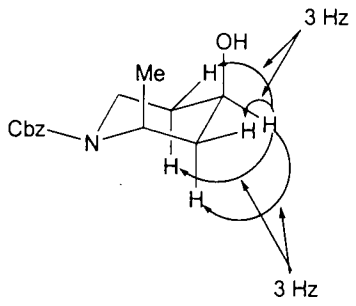


Figure 28: Coupling constants used in assignment of alcohol **72**

Product **73** on the other hand was found to be hydroxylated on position 3 of the heterocycle. The C*H*OH multiplet was not sufficiently resolved to ascertain the axial/equatorial positioning of the introduced hydroxyl group but nOe studies suggested that the methyl group lies on the same side of the piperidine ring (figure 29). If we assume again that the 2-methyl group lies axially, due to the $A^{(1,3)}$ strain, then the hydroxyl group would be situated equatorially.

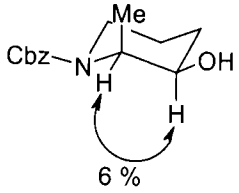


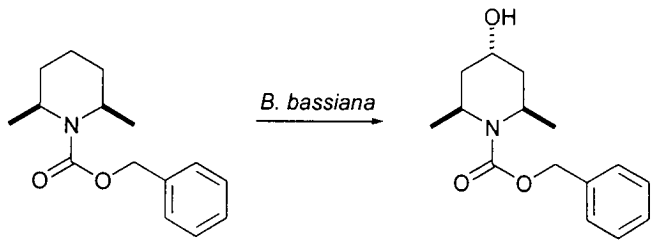
Figure 29: nOe enhancement used in assigning **73**

The results from this incubation are again largely analogous to those observed by Johnson, although in this case, the authors did observe a minor 4-keto product. Their identification of even a small amount of ketone is interesting since in no case did we identify further oxidation.

Incubation of N-Cbz-cis-2,6-dimethylpiperidine 74[74*]

When *N*-carboxybenzyl-*cis*-2,6-dimethylpiperidine **74** was incubated with *Beauveria bassiana*, one hydroxylated product was recovered. ^{1}H and ^{13}C NMR indicated this

compound to be the 4-hydroxy derivative **75** (figure 30), this product has relative configuration 2,6-*cis*-4-*trans* (again assuming that the 2-alkyl groups adopt an axial positioning) (figure 31).



74 **75**

Figure 30: Conversion of 2,6-dimethylpiperidine **74**

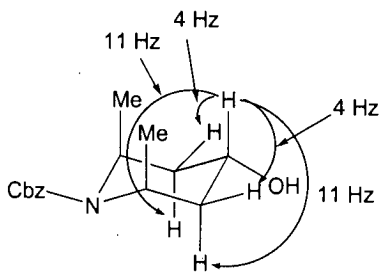
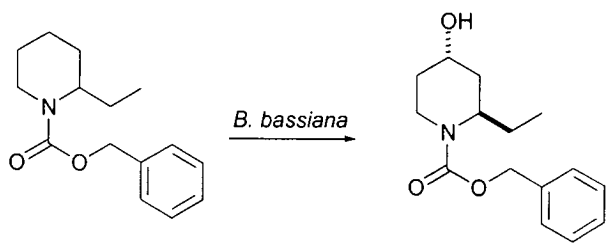


Figure 31: Coupling constants used in assignment of **75**

Incubation of N-Cbz-2-ethylpiperidine 76

N-Cbz-carboxybenzyl-2-ethylpiperidine **76** hydroxylation occurs almost exclusively at the 4-position to give the 4-hydroxypiperidine product **77** in good yield (figure 32).



76 **77**

Figure 32: Selective hydroxylation of **76** by *Beauveria bassiana*

From examination of the 1D proton NMR spectrum, the hydroxyl group was thought to lie in an equatorial orientation (figure 33). NOE studies added to this evidence showing the C*H*OH proton to be axial. The 2-ethyl group is again assumed to lie in an axial position.

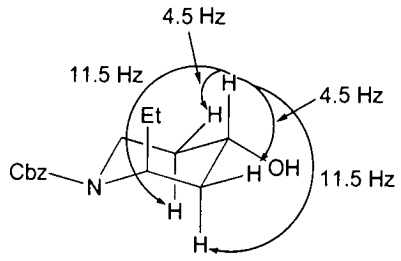
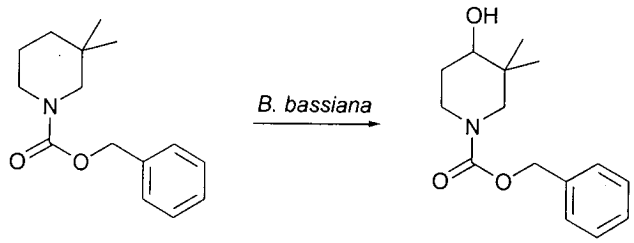


Figure 33: Coupling constants used in assigning hydroxylated product **77**

Two further substrates have been tested with the fungus, which do not correlate with the substrates used by Johnson, but add to the overall series and the investigation of the tolerance of the active site to changes in the substrate.

Incubation of N-Cbz-3,3-dimethylpiperidine 78

Incubation of the 3,3-dimethylpiperidine derivative **78** with the fungus provided the 4-hydroxylated product **79**, with the hydroxyl group introduced equatorially (figure 34). It was also noted that the rate of this hydroxylation was enhanced and that no substrate remained after two days, whereas other reactions required three days for complete substrate disappearance.



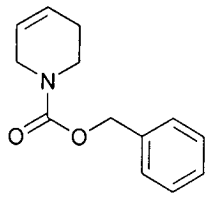
78 **79**

Figure 34: Transformation of dimethyl substrate **78**

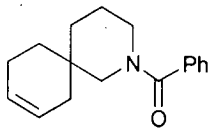
Interestingly, this result was another case where no hydroxylation of the alkyl group was observed, unlike those substrates employing the *N*-benzoyl aromatic side chain where, in more than one case, the alkyl group was hydroxylated.

Incubation of N-Cbz-2,3,6-trihydropyridine 80

The only result from this series that was somewhat unexpected was obtained when the fungus was challenged with the unsaturated piperidine derivative, *N*-carboxybenzyl-2,3,6-trihydropyridine **80** where no biohydroxylation was noted and unreacted starting material was recovered. This result was surprising since the 3,4-alkene was envisaged to be ideally placed for epoxidation and because cytochrome P-450 monooxygenases, in particular those of *Beauveria bassiana*, are known to produce epoxides from alkenes such as **81** and **82**.[80,81]



80



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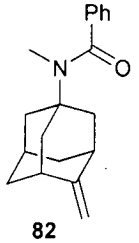


82

2.4 Discussion

Degradation of substrate/hydroxylated product

The Cbz-protecting group is potentially labile to other enzymes in the fungus and was a major concern when employing this series of substrates with the whole cell system. Microbial carbamate hydrolases have been reported in whole cell systems[27] and degradation of alkyl substituted aromatics by benzylic hydroxylation in fungi has been well studied.[82] Both of these degradation pathways were potential problems with our substrate design.

The only case in which these problems arose was *N*-Cbz-2,6-dimethylpiperidine **74** where the hydroxylated product **75** was isolated in very low yield (~10 mg from 270 mg substrate), but a large amount of benzoic acid was also recovered (~90 mg). The isolation of benzoic acid suggests some sort of benzylic hydroxylation, either of the starting material or of the hydroxylated product. Such a degradative mechanism would not be possible in the *N*-benzoyl case and is reflected in the higher yield reported.

The yields of product recorded in these transformations are between 5 – 48 %, which is very respectable for this type of reaction (table 1).[33,35,36] Unlike many literature examples, no starting material was recovered along with the products after three days incubation. It is likely that the mass balance of the conversions (greater than 50 % of substrate unaccounted for) is lost through degradative mechanisms.

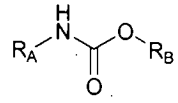
Substrate	Substituent	Yield[†]
57	None	33
62	4-methyl	45
67	3-methyl	24
71	2-methyl	14
74	2,6-dimethyl	5
76	2-ethyl	45
78	3,3-dimethyl	48

Table 1: Yields of biotransformations reported for substituted *N*-Cbz-piperidines

It has previously been reported that the inclusion of 'inverted' urethane linkers in substrates for *Beauveria bassiana* led to no biotransformation whatsoever; substrates of structure **83** where R_A is aryl and R_B alkyl are successfully hydroxylated whereas the 'inverted linker' where R_A is alkyl and R_B is aryl are not. Based on these results, it was

[†] Combined yields taken before separation of hydroxylated products

proposed that conjugation between the aromatic ring and the carbamate carbonyl is necessary for biotransformation to occur.[29]



83

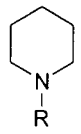
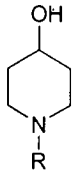
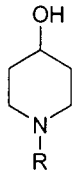
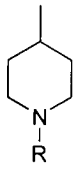
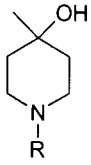
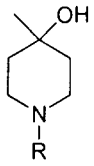
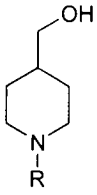
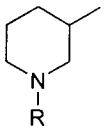
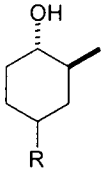
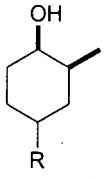
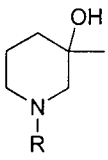
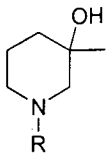
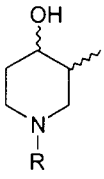
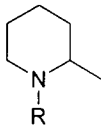
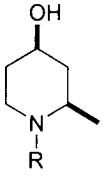
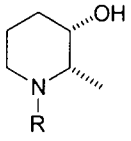
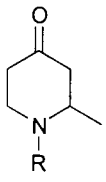
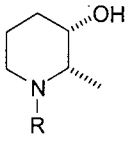
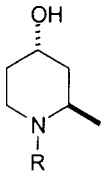
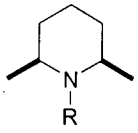
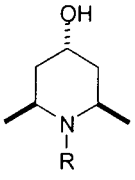
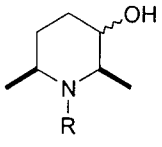
Our observations however are contrary to these results and clearly show that conjugation between the aromatic moiety and the carbonyl is not a prerequisite for hydroxylation.

Regioselectivity – effect of alkyl substitution

The change in regioselectivity of hydroxylation around the piperidine ring from the 4- to the 3-position with regioisomeric alkyl substitution is difficult to rationalise in terms of steric restrictions. For piperidines **57** and **62**, access to the favoured 4-position by the hydroxylating species is unrestricted, but methyl substitution at the 2- and 3-position must move the 4-position sufficiently far from this species that hydroxylation also occurs at the 3-position. One might expect that extending the 2-alkyl chain would therefore result in even more pronounced selectivity for the 3-position. However, *N*-carboxybenzyl-2-ethylpiperidine **76** yielded only the 4-hydroxylated compound **77**. Although the effect of alkyl substitution is difficult to rationalise, it is noted that small changes in substitution can make significant differences to the regioselectivity. Molecular modelling of the substrates may provide further insight into the effect of substitution.

Regioselectivity – effect of aryl side chain

One of our key aims in this investigation was to gain more understanding of the factors involved in the regioselectivity. In order to understand these, it is important to compare our observations with those previously reported by the Upjohn research group.[33]

Substrate	Products R = $PhCH_2OCO$	Products R = PhCO
 **57**	 **58**	
 **62**	 **63**	 
 **67**	   **68** **69** **70**	 
 **71**	  **72** **73**	  
 **74**	 **75**	

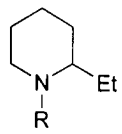
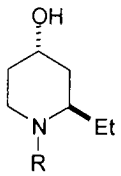
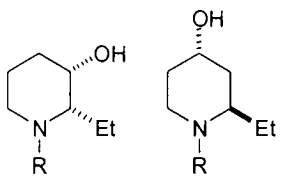
Substrate	Products R = $PhCH_2OCO$	Products R = PhCO
 **76**	 **77**	

Table 2: Results of biohydroxylations of Cbz-alkyl piperidines including a comparison with the results obtained from the *N*-benzoyl analogues[33]

The results obtained are compared in table 2 and can be summarised:

(i) Cbz series shows selectivity for 4- position, whereas benzoyl series shows product mixtures in most cases.

(ii) Cbz series shows no side chain hydroxylation – common in benzoyl series

(iii) Cbz series shows no further oxidation to ketone – noted in benzoyl series

To compare and draw tentative conclusions from these observations, we have to assume that the same hydroxylase enzyme within the whole cell system carries out all the biohydroxylations reported. Although there is clearly more than one active hydroxylase in the system, the assumption is thought to be valid given the close structural similarities of the substrates.

With this assumption in mind, it is difficult to explain the improvement in regioselectivity realised on examination of the Cbz alkylpiperidine series solely on the basis of the current active site model. Even in the updated models, it is assumed that the key restraint on the site of hydroxylation is the distance between the electron rich moiety and the hydroxylation site.

The hydroxylase is clearly now disposed to hydroxylation in the 4-position, except where there is a 2- or 3-methyl substituent when the selectivity is compromised, suggesting that the active site of the *Beauveria* hydroxylating system is very sensitive to relatively small changes in the nature of the protecting group. The distance from the carbonyl group to the putative site of hydroxylation is, of course, unchanged from *N*-benzoyl to *N*-benzyloxycarbonyl. Our results would suggest that the more critical characteristic for selective hydroxylation is the distance from the carbonyl to the aromatic binding pocket.

In addition, greater flexibility of the *N*-carboxybenzyl molecule is afforded by rotation around the benzylic centre. This would appear to allow improved accommodation by the active site and perhaps greater regioselectivity for some substrates.

We propose that there is a defined aromatic binding pocket within the active site of the hydroxylase and that interaction of the substrate with the enzyme is affected sufficiently for the hydroxylation site to be no longer controlled solely by the site of the electron rich moiety in the substrate molecule.

Stereochemistry of hydroxylation

It has been extensively reported that, in rigid systems, the hydroxyl group is introduced *trans* with respect to the amide functional group.[35,81,83,84] By the use of proton NMR analysis, we have established that the hydroxyl group is introduced into the equatorial position in most cases. The exceptions are the 2-methyl-4-hydroxyl product **72** where the hydroxyl group was seen to lie in an axial position (this was shown by the lack of an nOe enhancement between the axial C(6)H and the C(4)H and also from the coupling constants of the C*H*OH signal, showing no axial-axial coupling) and the 3-methyl-4-hydroxyl product (the acetylated derivative of which was isolated **69a**) where the C*H*OAc signal showed no axial-axial coupling. Our results therefore show a clear

preference for hydroxylation *trans* to electron rich carbamate. It is unclear whether such selectivity exists in the *N*-benzoyl series due to assignment difficulties.

Stereoselectivity of hydroxylation

Unusually for flexible substrates, Johnson and co-workers reported some enantiodifferentiation by the *Beauveria* hydroxylating system when operating on *N*-benzoylalkylpiperidines.[33] On examination of the data presented, one must be sceptical as to the accuracy of these conclusions. The authors themselves note that their product isolation and purification depends on crystallisation and given that in some cases the crystallisation yields are as low as 6 % of the crude fraction, there is a likelihood that at least some resolution could have taken place. Although not all of the optically active products have crystallisation yields as low as this, the results should be examined critically. The optical purity of our products has not been determined but it was seen that in all cases, a 72 hour incubation of the substrate with the fungus resulted in complete substrate disappearance and since the yield of hydroxylated products in some cases was approximately 50 %, it is perhaps less likely that a resolution process is operating. It is likely, on the basis of previous work,[85-87] that the enantioselectivity and resulting enantiomeric excess (if relevant) will depend on many factors, including the time course of the reaction. There could be preferential hydroxylation of one enantiomer of the substrate at a single position as well as preferential metabolism of one of the product molecules, both of which would affect the enantiomeric excess of the final product. At this stage of the project and due to the expected low enantioselectivity with *Beauveria bassiana*, investigation of the optical purity of these products was not undertaken.

2.5 Conclusions

This study has established that the regioselectivity of hydroxylation by *Beauveria bassiana* ATCC 7159, an organism much used for biotransformations of this type, may

be significantly altered by changes to the linker between the putative site of hydroxylation to the aromatic group. This finding confirmed the previously noted observation that apparently small changes in substrate structure can significantly alter the regioselectivity of hydroxylation. In light of the comparison between the previously studied *N*-benzoylpiperidines and our *N*-benzyloxycarbonylpiperidines, it appears that regioselectivity is not only determined by the distance between hydroxylation site and carbonyl group that is constant in the molecules studied. We propose that the active site of the hydroxylase may contain a defined aromatic binding pocket and that the distance from aromatic group to site of hydroxylation may also be a contributing factor to the regioselectivity. The site of hydroxylation in a series of related substrates was found to be dependent on substitution of the heterocyclic ring and could be manipulated by appropriate alkyl substitution around the ring.

3 Biotransformations with Rhodococcus sp. NCIMB 9784

3.1 Aims

The use of *Rhodococcus* species in biotransformations has mainly been for nitrile hydrolysis, indeed this use has proven the utility of *Rhodococcus* systems for industrial scale biotransformation. Bioremediation tests have also shown the adaptability of *Rhodococcus* species to changing environments. Although examples do exist, *Rhodococcus* spp. are not widely used for oxidative biotransformations. It is known that many Actinomycetes such as *Streptomyces* spp. can be useful for biohydroxylations and our aim was to investigate the utility and scope of Actinomycetes belonging to the family *Rhodococcus* for such transformations. We were particularly interested in the hydroxylation of non-activated carbon centres by cytochrome P-450 monooxygenases.

The work described here was carried out as part of a larger investigation into the, as yet under utilised, activity of *Rhodococcus* species as biohydroxylation catalysts.

Initial screening, carried out by Dr. Gideon Grogan, encompassed six *Rhodococcus* spp., which were identified from the literature as containing hydroxylating monooxygenase (and therefore potentially cytochrome P-450) activity. The organisms were grown on carbon sources reported to elicit their monooxygenase hydroxylation activities and their hydroxylation activity tested against a number of substrates known to be hydroxylated by cytochrome P-450 monooxygenases.

One of the monooxygenase systems identified from this screening was *Rhodococcus* sp. NCIMB 9784. As highlighted in the introduction, this organism was reported to catalyse the regioselective hydroxylation of (+)-camphor.[66] To our knowledge, there have been no further literature reports of the use of this organism.

Our aim in studying this hydroxylating organism was to investigate its activity in terms of substrate specificity and selectivity. Studies within our group have shown that (+)-camphor is hydroxylated in the 6-*endo* position in a hydroxylation that is complementary to the 5-*exo* hydroxylation carried out by cytochrome P-450_{cam} from *Pseudomonas putida*. In light of this complementary stereoselectivity, we wanted to investigate the specificity and selectivity of the *Rhodococcus* system for comparison with the well-characterised cytochrome P-450cam system.

3.2 Growth of the organism

Since monooxygenase activities are often substrate-inducible,[57] the organism was initially grown on camphor in an effort to elicit the hydroxylation activity reported previously.[66] Growth on basal salts medium augmented with ferrous sulfate, to allow heme production, and (+)-camphor, as the sole carbon source, was found to be extremely slow. Faster, more reliable growth was obtained using starter cultures grown on sodium pyruvate as sole carbon source as the innoculum for larger scale growth on D-(+)-camphor. Using pyruvate-grown starter cultures, it was found that the bacteria grew well in both shake flasks and in a fermenter, yielding approximately 3 - 4 g L^{-1} wet cell mass. SDS-PAGE of a cell extract showed that growth on camphor induces four major proteins. Of these, one has a molecular weight of around 45, 000 Da,[68] which is known to be the typical mass of cytochrome P-450 enzymes. For comparison, cells grown on sodium pyruvate as a general carbon source were also investigated.

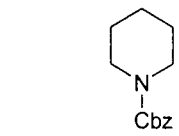
3.3 Methods

Biotransformations using the *Rhodococcus* species under investigation were carried out using resting cells. Such transformations are carried out as whole cell systems but unlike growing whole cells, there are no nutrients present for cell growth. The organism

is grown to secondary growth phase and the cells harvested and washed. The cells are then re-suspended in around one-tenth growth volume of buffer ready for transformation. The substrate was again added as a solution in ethanol to a final concentration of between 0.1 and 0.5 mg mL^{-1}. The use of resting cells can be advantageous since purification is often simpler than for growing cell transformations.

3.4 Oxidation of non-camphor-like substrates

In order to investigate the hydroxylating ability of the organism, small scale biotransformations were carried out using a range of substrates.[68] *N*-carboxybenzylpiperidine **57**, 2-methyl-5-benzyloxymethyl-tetrahydrofuran **84**, indole **85**, cumene **86** and benzofuran **87** were seen to be unchanged on incubation with a resting cell suspension of *Rhodococcus* sp. NCIMB 9784.



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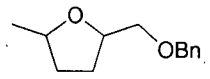

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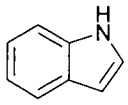

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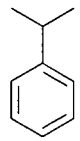

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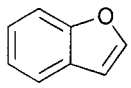
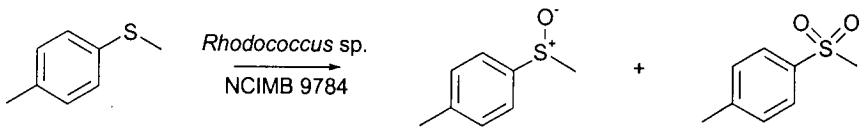


87

These substrates had previously been reported to be hydroxylated by cytochrome P-450 monooxygenases from various bacterial and fungal sources, but were not hydroxylated by this organism.

Methyl-*p*-tolyl sulfide **88** was seen to be transformed by sulfoxidation to give a mixture of products (figure 35). Although this result did show hydroxylating abilities of the

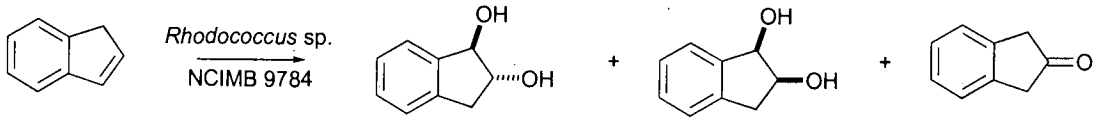
organism, the transformation could have been catalysed by monooxygenase, dioxygenase or other oxidase activity.



88 89 90

Figure 35: Sulfoxidation by *Rhodococcus* sp. NCIMB 9784

The most interesting results from the initial testing were that tetralin (1,2,3,4-tetrahydronaphthalene) **91** and indene **92** were both transformed into products distinct from their starting materials. Indene **92** was converted into four products, three of which could be identified from commercially available standards, as indicated in figure 36.



92 93 94 95

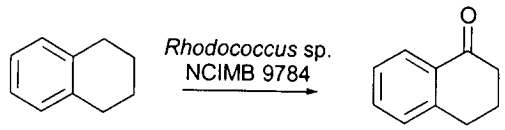
Figure 36: Biotransformation of indene **92**

This transformation of indene is potentially important since *trans*- **93** and *cis*-indandiol **94** are both potential synthons for Crixivan **46** (as discussed in section 1.5.3). Two other *Rhodococcus* species have recently been reported to have similar biocatalytic activity and to carry out this transformation.[56]

The transformation of tetralin **91** to one product instigated a fuller investigation because it is known that tetralin is transformed to (R)-1-tetralol by cytochrome P-450_{cam}[88] and it was hoped that the transformation of this substrate might provide an interesting comparison between the two P-450 systems.

Tetralin

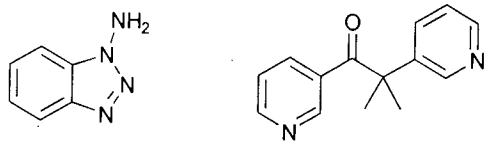
A larger scale biohydroxylation of tetralin was carried out to investigate the transformation further, leading to identification of the product as α-tetralone **96** (figure 37). Our interest in hydroxylation by cytochrome P-450 monooxygenases prompted us to ascertain whether this transformation was carried out by a cytochrome P-450 monooxygenase or some other oxidising enzyme in the organism.



91 **96**

Figure 37: Selective oxidation of tetralin **91**

The action of a cytochrome P-450 monooxygenase can be detected using an inhibition assay. Although this indirect evidence is not unequivocal, in the absence of the isolated enzyme for characterisation it can be regarded as support for cytochrome P-450 action. Two common inhibitors used for cytochrome P-450 monooxygenases are 1-aminobenzotriazole **97** and metyrapone **98**. 1-Aminobenzotriazole **97** is a benzyne precursor that can strongly inactivate cytochrome P-450 monooxygenases due to the formation of an adduct between benzyne and two of the vicinal nitrogens of the porphyrin ring in the P-450.[27] Metyrapone **98** can inhibit the action of cytochrome P-450 monooxygenases by donating from one of its nitrogens to the haem centre in the cytochrome, rendering it catalytically inactive.[89]



97 **98**

Analysis of incubations of tetralin **91** with *Rhodococcus* sp. NCIMB 9784 in the presence of inhibitors **97** or **98** showed no product. Since these two inhibitors act by

different modes of action, these studies were regarded as strong evidence that a cytochrome P-450 was operating in the biotransformation of tetralin.

Tetralin like substrates

Due to the successful transformation of tetralin, a number of commercially available analogues were tested as potential substrates in order to gain information about the specificity of the organism.

Both α- **99** and β-tetralol **100** were turned over by *Rhodococcus* sp. NICMB 9784, with α-tetralone **96** identified as the product of the α-tetralol transformation (figure 38). Although the product of the β-tetralol **100** transformation was not isolated and identified, we speculate that it is the corresponding β-ketone on the basis of its similarity to α-tetralone.

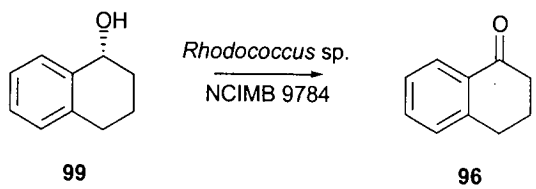


Figure 38: Biotransformation of α-tetralol **99**

Indan **101** was found to be converted to give 1-indanone **102** in the analogous transformation to that of tetralin, *via* benzylic oxidation (figure 39).

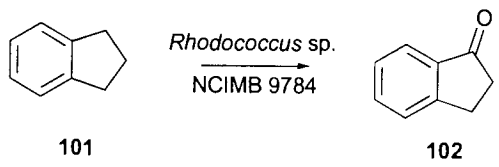
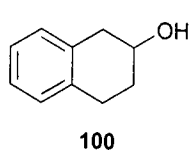


Figure 39: Production of indanone by *Rhodococcus* sp. NCIMB 9784

A number of other related structures were tested as substrates for the organism with little success. Decalin (bicyclohexyl) **103**, the fully saturated derivative of tetralin, was not transformed, suggesting that the aromatic ring is important and perhaps that the organism needs the more reactive benzylic centre to be present for successful oxidation. Naphthalene **104**, the fully unsaturated derivative is also fully recovered from the biotransformation mixture showing that the organism does not appear to catalyse aromatic hydroxylation. 1,2-dihydronaphthalene **105** was found to be hydroxylated to a mixture of many products, probably arising from epoxidation, benzylic hydroxylation and dihydroxylation. Incubation of α-tetralone **96** for longer periods showed that no further transformation took place.



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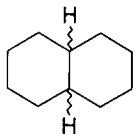

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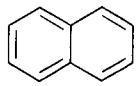

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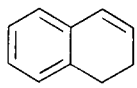

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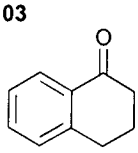


96

These studies have, unfortunately, shown that the oxidation capabilities of *Rhodococcus* sp. NCIMB 9784 are limited; oxidative biotransformation seems to work best on benzylic carbon centres or on alkene regions of the substrates and in no case was hydroxylation of non-activated carbon centres noted.

Tetralin biotransformation mechanism

It seems likely that the transformation of tetralin by *Rhodococcus* sp. NCIMB 9784 consists of more than one step and we have already shown that α-tetralol **99** is transformed by the resting cell system to produce α-tetralone **96**. It is therefore proposed that initial hydroxylation of tetralin **91** is catalysed by a monooxygenase system yielding an alcohol, which is then transformed to the ketone by an alcohol dehydrogenase (ADH) (figure 40).

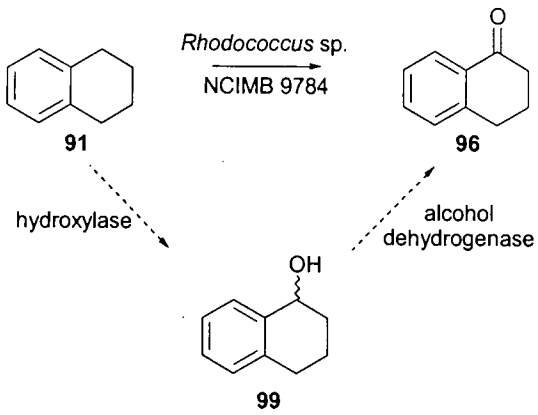


Figure 40: Proposed route to α-tetralone from tetralin

The transformation of **91** was followed by GC analysis and an approximately linear rate of substrate utilisation and product appearance with time was noted with little trace of the alcohol observed (figure 41). This demonstrates that, if produced as an intermediate in the transformation, the alcohol is transient.

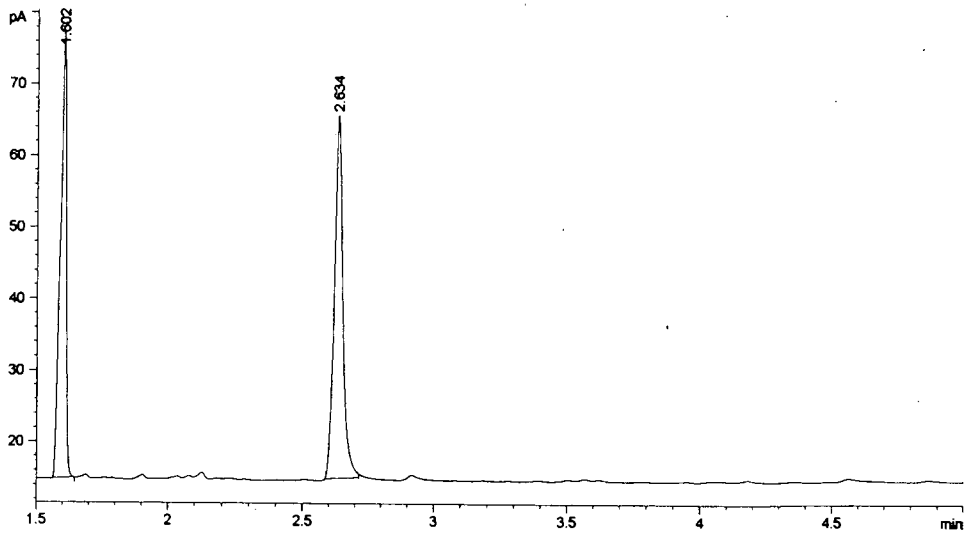
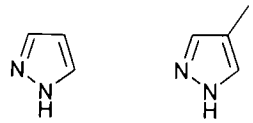


Figure 41: Gas chromatograph of tetralin **91** transformation showing substrate (1.6 mins) and product (2.6 mins) (oven temperature 160 °C, injector temp 220 °C, FID temp 300 °C)

The proposed route can be tested by inhibition of alcohol dehydrogenases. The most widely reported alcohol dehydrogenase inhibitors are pyrazole **106** and 4-methylpyrazole **107**.[90] Unfortunately both of these compounds contain a nitrogen atom, which could potentially donate to the haem centre, and therefore inhibit cytochrome P-450 monooxygenases.



106 **107**

Alcohol dehydrogenase inhibition studies were carried out similarly to the cytochrome P-450 inhibition studies described previously. These inhibition studies indicated that while both 1-aminobenzotriazole **97** and metyrapone **98** had no effect on the transformation of the tetralols, 4-methylpyrazole **107** inhibited the turnover of α-tetralol **99** by around 10 %. This result indicated that although no cytochrome P-450 was taking part in the transformation, it is likely that an alcohol dehydrogenase was involved.

On incubation of tetralin **91** with 4-methylpyrazole in a resting cell culture of the bacteria no product was detected. This result was taken to confirm the hypothesis that 4-methylpyrazole **107** would not only inhibit alcohol dehydrogenases in the system, but would also inhibit any P-450's present making it extremely difficult to prove whether tetralin **91** is converted to the tetralone **96** *via* the tetralol **99**.

It is interesting to note that if this is the route to the product, the transformation carried out by the cytochrome P-450 in this *Rhodococcus* sp. is the same as that carried out by cytochrome P-450$_{cam}$.[88] This would be significant since it would suggest that while the camphor hydroxylases from *Pseudomonas putida* and *Rhodococcus* sp. NCIMB 9784 show differing regioselectivity in their hydroxylation of camphor, they can hydroxylate at the same position of other substrates.

Nature of tetralin hydroxylase

At this stage, it was unclear whether the transformation of tetralin and related substrates was being carried out by a cytochrome P-450 and if it were, whether it was the camphor utilising P-450 or another previously unidentified P-450 in the organism. In order to investigate this, cells grown on sodium pyruvate as sole carbon source were tested as biocatalysts to compare with previous results where the cells utilised were grown on D-(+)-camphor. It was found that tetralin was completely transformed by the pyruvate grown cells. This suggested that the transformations may have been catalysed by a constitutively expressed hydroxylase, rather than by the camphor hydroxylating cytochrome P-450.

Binding assays were carried out with crude extracts of camphor grown cells in an attempt to prove this theory. These rely on the shift of the Sorét maximum of the cytochrome P-450 on addition of a substrate which binds to the enzyme.[91] The Sorét maximum depends on the environment of the iron centre, which is surrounded by the porphyrin group and an axial cysteine ligand. The Sorét maximum lies at 417 nm for the unbound protein where water acts as the sixth ligand to the haem centre (solid line) and 392 nm for the bound state where the water is displaced (dotted line).

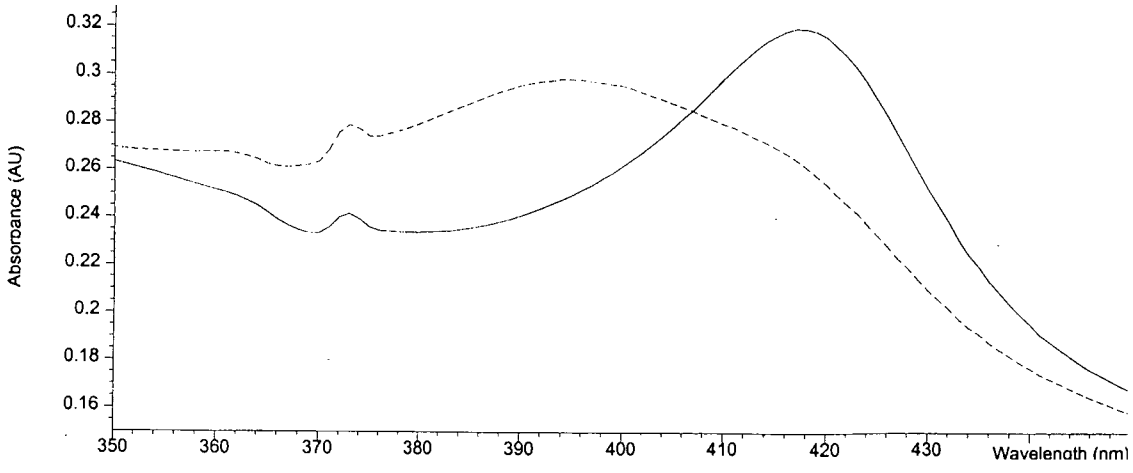


Figure 42: Camphor binding assay

Camphor was seen to cause a complete shift from 420 nm to 390 nm (figure 42) whereas tetralin showed no obvious shift (figure 43).[‡] This lends credibility to the theory that the tetralin transformation may be carried out by a cytochrome P-450 but not that which is responsible for the hydroxylation of camphor.

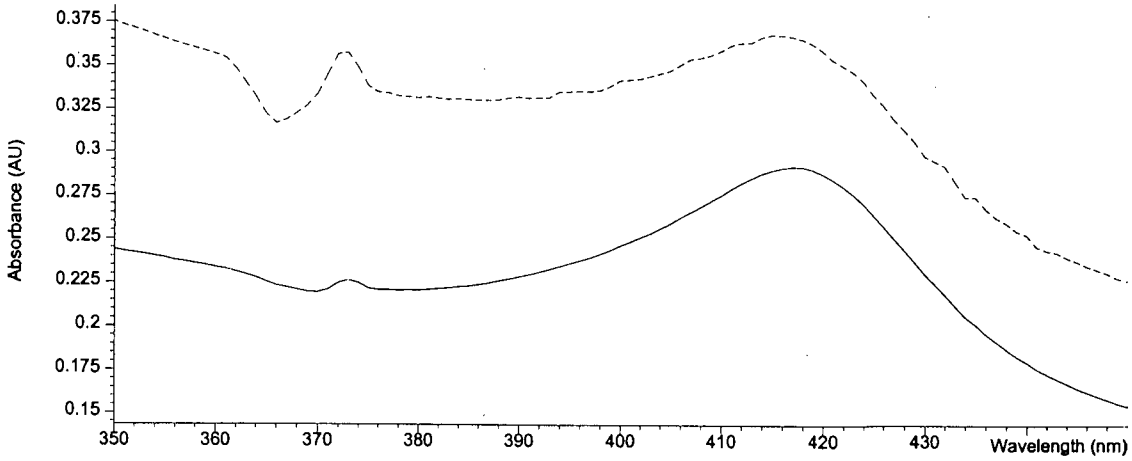


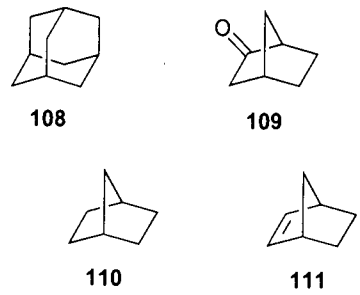
Figure 43: Tetralin binding assay: solid line protein only, dotted line protein plus tetralin

3.5 Metabolism of camphor-like substrates

Our initial aim for the investigation of *Rhodococcus* sp. NCIMB 9784 was to investigate the utility of its cytochrome P-450 monooxygenases. It has been shown that tetralin and related substrates are hydroxylated, seemingly by a cytochrome P-450 but not that which hydroxylates camphor. We were therefore interested in investigating the scope of reaction of the camphor hydroxylase and chose to study the hydroxylation and metabolism of substrates related to camphor. The terpenoid substrates chosen were adamantane **108**, norcamphor **109**, norbornane **110** and norbornylene **111**. These were chosen primarily due to their structural similarity with camphor but also because the likely hydroxylation products are all commercially available. It was found however that

[‡] Due to the lower solubility of tetralin in the aqueous buffer, a slight shift may not have been obvious

hydroxylation of these substrates was difficult to detect. It is likely that this is due to further metabolism of the products through the camphor degradation pathway. Due to these difficulties, further work on the camphor hydroxylase in its whole cell form was suspended and the characterisation of substrate specificity was continued using the isolated camphor hydroxylase enzyme system where further metabolism is no longer an issue.



108 **109**

110 **111**

Binding assays were also carried out with these substrates, with the addition of an ethanol solution of substrate to the crude enzyme extract from camphor grown cells and any shift in the Sorét maximum noted. All of the substrates tested showed some shift, with norcamphor **109** in particular showing a Sorét band shift (figure 44). This indicated that the camphor-like substrates bind to the camphor hydroxylating cytochrome P-450.

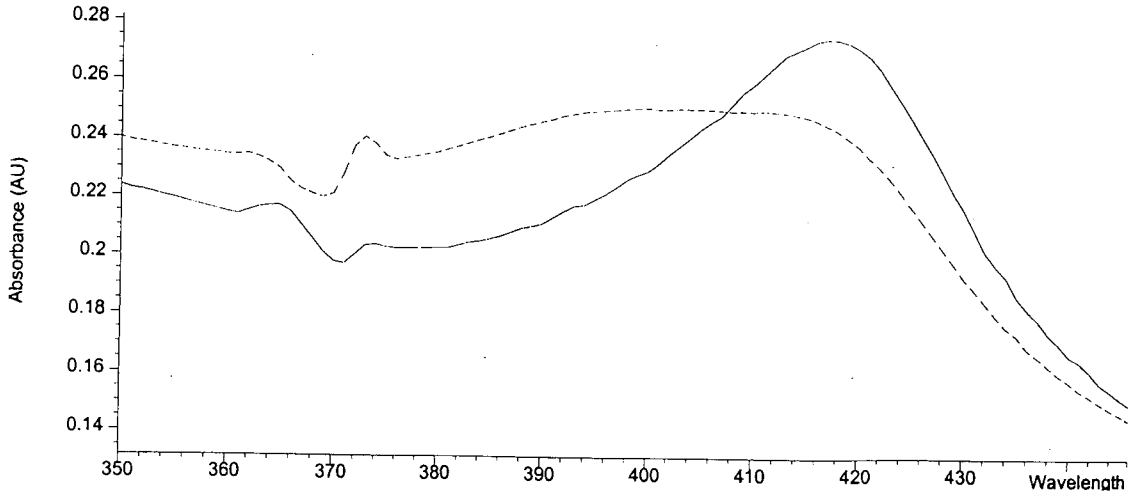


Figure 44: Norcamphor binding assay

Because these binding assays showed some binding to the camphor-hydroxylating enzyme, they lend evidence to the supposed hydroxylation of camphor-like substrates by the enzyme.

3.6 Conclusions

Our conclusions from these studies are two fold. Although no good substrates were positively identified for the camphor utilising cytochrome P-450 that initially attracted our attention to this organism, we believe that we have identified a second cytochrome P-450 in the system. We have discovered that this cytochrome P-450 has narrow substrate specificity for tetralin **91** and related substrates. We believe that the oxidation to a ketone occurs *via* the alcohol in a two step procedure catalysed by a cytochrome P-450 and an alcohol dehydrogenase. It is also noted that hydroxylation of substrates seems to depend on the existence of a benzylic or alkene carbon within the molecule. Thus, the outcome of the tetralin incubation was not particularly interesting in the context of microbial hydroxylation of non-activated carbon centres. The transformation of indene **92** to yield, amongst other products, indandiols **93** and **94** is potentially interesting as there is known synthetic utility for such compounds in their optically pure form.[56]

From our preliminary studies into the camphor utilising hydroxylase, it would seem that, like cytochrome P-450$_{cam}$, the camphor monooxygenase in *Rhodococcus* sp. NCIMB 9784 has narrow substrate specificity, limited to camphor-like structures. Camphor analogues such as norcamphor **109** were found to bind to the enzyme, as indicated by Sorét band shift assays. Although this may limit its use as a hydroxylation catalyst, the enzyme has now been isolated and could potentially be cloned for use in a suitable host system for hydroxylation. The enzyme could also be investigated in a manner similar to

cytochrome P-450_{cam} where mutagenesis experiments have widened the substrate range such that larger molecules containing aromatic groups can now be hydroxylated by cytochrome P-450_{cam}.[92,93]

4 Biotransformations with Rhodococcus rhodochrous NCIMB 9703

4.1 Aims

As indicated in chapter 3, we are interested in the potential utility of *Rhodococcus* species as biohydroxylation catalysts, a property that has, as yet, not been fully investigated. Screening of a range of such microorganisms thought to have potentially cytochrome P-450 dependent hydroxylating abilities identified a number of organisms that warranted further investigation. One of these, *Rhodococcus* sp. NCIMB 9784, is reported in chapter 3 where we have shown that the system appears to contain at least two cytochrome P-450 monooxygenases which both show narrow substrate specificity.

Another of the *Rhodococcus* species identified for further investigation was *Rhodococcus rhodochrous* NCIMB 9703. As highlighted in the introduction, this organism is reported to catalyse the terminal hydroxylation of *n*-octane. To our knowledge there have been no investigations as to the utility of this organism as a general biohydroxylation catalyst.

Our aim in this investigation was to determine the existence of cytochrome P-450 monooxygenase(s) within the organism and to investigate the scope and selectivity of any hydroxylation catalysed.

4.2 Growth of organism

Rhodococcus rhodochrous NCIMB 9703 is reported to contain an inducible NADH dependent hydroxylase that catalyses the conversion of *n*-octane to 1-octanol.[48] Growth

of the organism on octane was therefore attempted to induce this hydroxylating enzyme but growth on basal salts medium augmented with ferrous sulfate and *n*-octane was found to be extremely sluggish. In common with *Rhodococcus* sp. NCIMB 9784, it was found that initial growth on sodium pyruvate for use as innoculum for larger scale growth on *n*-octane proved a successful and reliable method for growth. Due to the potential experimental risks when utilising a flammable additive such as octane,[94] fermenter growth was not attempted but growth in shake flask was found to yield sufficient cell mass for biotransformation. For comparison, cells were also grown on sodium pyruvate with no induction. Cell mass was typically greater from growth on pyruvate (5.5 gL^{-1}) compared with growth on octane (2.5 gL^{-1}).

4.3 Methods

Biotransformations using *Rhodococcus rhodochrous* NCIMB 9703 were carried out using resting cell suspensions where the cells are live but do not have the nutrients to continue growing. As for *Rhodococcus* sp. NCIMB 9784, it was envisaged that the use of resting cells might ease purification.

The organism was first grown to secondary growth phase and the cells harvested and washed. The cells were then re-suspended in approximately one-tenth growth volume of phosphate buffer (50 mM, pH 7.0) and substrate added as a solution in ethanol to a final concentration of 0.5 gL^{-1}. On completion, the cells were removed by centrifugation and the products isolated, purified by flash chromatography and identified by NMR spectroscopy.

4.4 Initial screening

Initial screening of the octane grown organism demonstrated that Cbz-piperidine **57** was oxidised selectively in the 4 position (figure 45), in a transformation analogous to that by *Beauveria bassiana* ATCC 7159.[73]

57 **58**

Figure 45:Transformation of **57** by *Rhodococcus rhodochrous* NCIMB 9703

This selective hydroxylation of a non-activated carbon centre was encouraging and prompted us to challenge the octane-grown organism with a variety of new substrates. Oxygen heterocycles were thought to be particularly interesting since, on deprotection, they would yield potentially useful polyfunctionalised molecules.

4.5 Synthesis of substrates

Benzyloxy substituted oxygen heterocycles were identified as potential substrates and a range of such substrates were synthesised.

2-Benzyloxy-tetrahydrofurans and pyrans were synthesised from the 2-hydroxy heterocycle by treatment with benzyl bromide in the presence of sodium hydride (figure 46) their carbocyclic analogues were also prepared *via* this route.

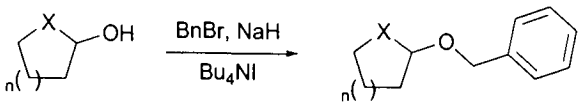
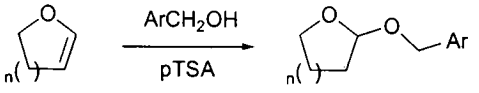


Figure 46: General benzylation method, X = C or O; n = 1 or 2

Substituted aryl derivatives were prepared by treatment of the dihydropyran or furan with the corresponding alcohol in the presence of *p*-toluenesulfonic acid (figure 47).



n = 1 or 2; Ar = Ph, C_6H_4Cl, $C_6H_4NO_2$, C_6H_4Me.

Figure 47: Preparation of substituted acetals:

The substrates were all produced in moderate to good yields in unoptimised reactions.

4.6 Biooxidations of tetrahydropyranosyl derivatives

General determination of regio- and stereochemistry

In most cases, the regiochemistry and relative stereochemistry of the products could be determined by NMR using coupling constants, COSY and nOe/NOESY experiments. In all cases, it was seen that the C2-H proton was present showing two couplings consistent with coupling to two C3 protons suggesting that they too were unaltered by the hydroxylation. This left C4, C5 and C6 as the potential hydroxylation sites. Of these, only C6 protons have easily identifiable signals that are shifted downfield due to their proximity to the ring oxygen. These were important signals since, on comparison to the starting material, they were simplified by hydroxylation, indicating that one of their coupling partners had been removed (figure 48). COSY experiments confirmed that the C*H*OH proton is coupled to the C6 protons and is therefore attached to C5.

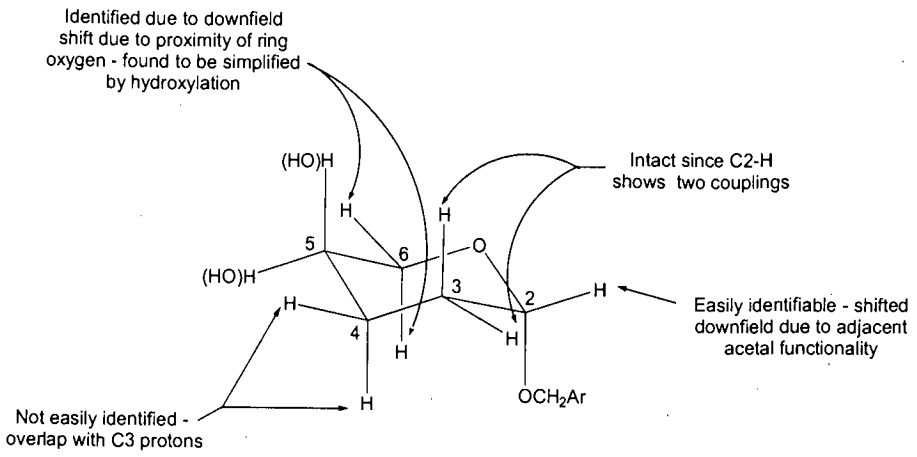


Figure 48: Important features for stereochemical assignment from NMR analysis

In general, the two products were identified as the 5-axial and 5-equatorial-hydroxylated compounds due to the coupling patterns of the C6-H signals. In the case of the 5-axial-hydroxy product, the C6-H*eq* signal typically showed three or four couplings: one geminal, one equatorial/equatorial and one or two fine W couplings to equatorial protons around the ring. Additionally the C6-H*ax* showed one axial/equatorial and one geminal coupling (figure 49).

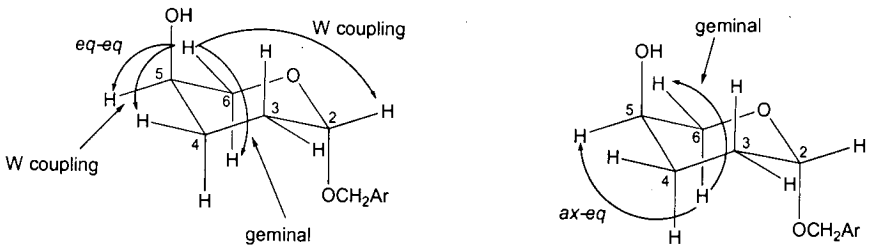


Figure 49: Typical coupling patterns of hydroxylated products

In the case of the 5-equatorial-hydroxy product, the C6-H*eq* showed coupling to its geminal partner, one axial proton and one or two fine W couplings and the C6-H*ax* showed two large couplings; a diaxial coupling and a geminal coupling (figure 50).

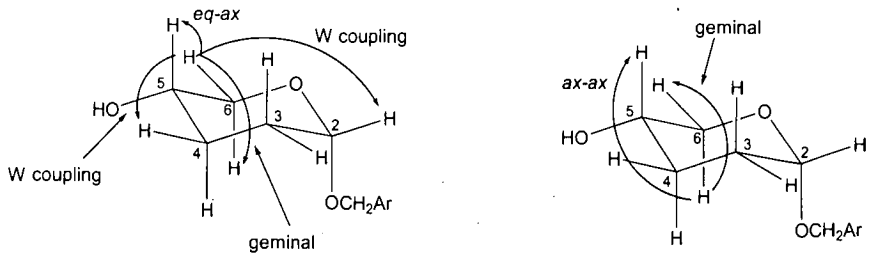


Figure 50: Typical coupling patterns of hydroxylated products

The C5-H signal, when sufficiently resolved, could also be used to identify the stereochemistry of the introduced hydroxyl group. Where 360 MHz ^{1}H NMR did not resolve the coupling patterns, nOe experiments were used to determine the relative stereochemistry around the ring.

Incubation of 2-(benzyloxy)tetrahydropyran 112

In the case of the unsubstituted substrate **112**, an inseparable mixture of hydroxylated products was obtained upon incubation with *Rhodococcus rhodochrous* NCIMB 9703 which was thought to be a mixture of 5-hydroxylated products. A small amount of one of these products was isolated but unfortunately, due to overlapping signals and unresolved multiplets, its identity could not be unequivocally determined. Acetylation of the product mixture failed to enable separation of the two compounds. The NMR spectrum did show that the C2 acetal group was in the axial position with the geminal proton showing only two small couplings and no axial-axial coupling. This axial positioning was noted throughout the series and was easily explained as a consequence of the anomeric effect.

Substrate **112** has been incubated with other biocatalysts under study within our laboratories with interesting results: incubation with isolated enzyme preparations of cytochrome P-450$_{cam}$ (Y96A mutant) yielded no isolable hydroxylated products, whereas incubation with growing cell suspensions of the fungus *Beauveria bassiana* ATCC 7159 yielded a complex mixture of products.[95] These results showed that the

Rhodococcus species under investigation has interesting complementarity with existing biocatalysts (figure 51).

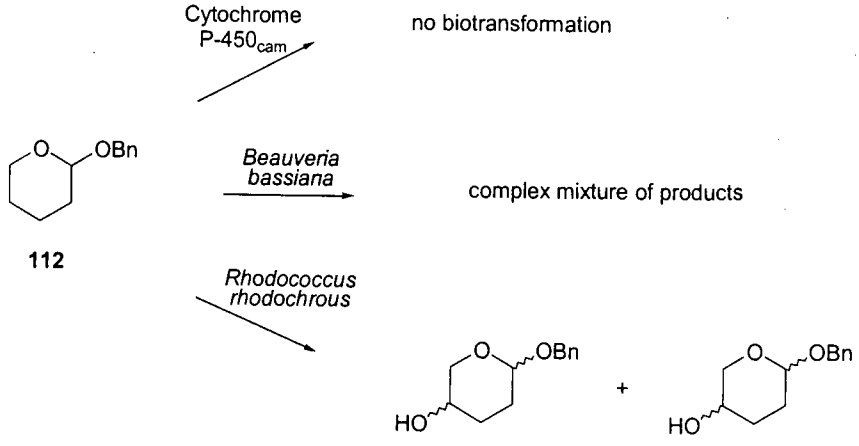
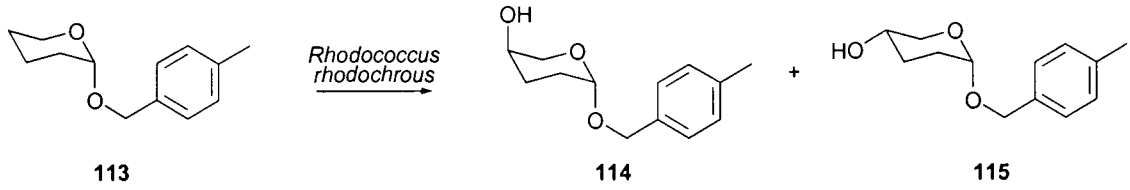


Figure 51: Incubation of **112** with different whole cell biocatalysts

Due to the difficulties in separating the products, this result was not further investigated and instead a range of analogues were examined. It has been shown previously[36] that the introduction of substituents on the aryl ring of substrates can direct hydroxylation away from the substituted position. Although hydroxylation of the aromatic ring was not noted in the biotransformation of acetal **112**, the incorporation of *p*-substituents was investigated to establish any directing or yield effect.

Incubation of 2-(4-methylbenzyloxy)tetrahydropyran 113

On incubation of the *p*-methyl substituted acetal **113** with *Rhodococcus rhodochrous* NCIMB 9703, the two products were identified as the 5-axial **114** and 5-equatorial hydroxy **115** compounds (figure 52). The products were identified on the basis of ^{1}H-coupling patterns in their ^{1}H NMR spectra.



113 **114** **115**

Figure 52: Transformation of methyl-substituted compound **113**

The ^{1}H NMR spectra initially showed overlapping signals but acquiring the spectrum in *d*6-benzene rather than *d*-chloroform was found to resolve the important signals sufficiently to allow coupling patterns to be examined (figure 53).

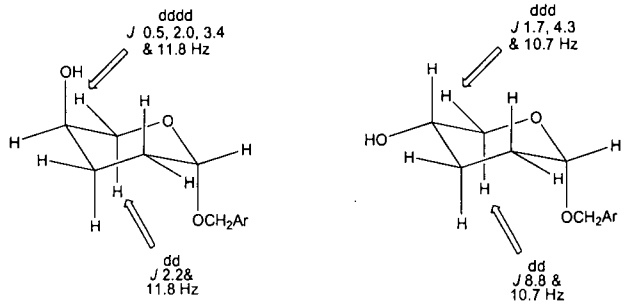
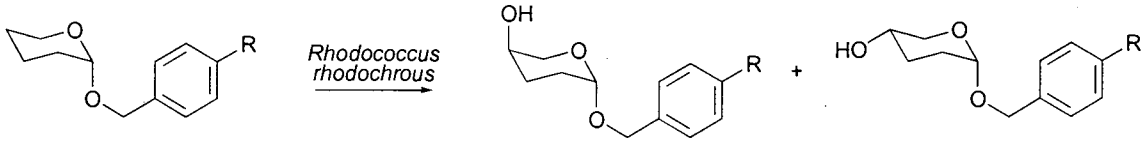


Figure 53: Coupling constants used in the identification of products **114** and **115**

Incubation of 2-(4-chlorobenzyloxy)tetrahydropyran 116[96]

Hydroxylation of the *p*-chloro analogue **116** showed little difference in regioselectivity to the 4-methyl substituted analogue **113** with the 5-hydroxy compounds **117** and **118** identified by analogy to the methyl-substituted products (figure 54).



116 R = Cl
119 R = NO_2

117 R = Cl
120 R = NO_2

118 R = Cl
121 R = NO_2

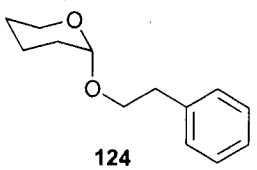
Figure 54: Transformation of tetrahydropyranosyl derivatives **116** and **119**

longer sited axially. The equatorial arrangement of the ether chain suggested that the hydroxyl group was introduced *trans* to the existing substituent on the ring.

At this stage it was unclear whether the gain in regioselectivity for ether **122** was due to the increased length of the ether linkage or due to its spatial orientation. It had been assumed thus far that the ether sits in an axial position on the ring due to the influence of the anomeric effect. In the case of substrate **122**, the insertion of the methylene cancelled the anomeric effect so that the ether sits in the sterically favoured equatorial position.

Incubation of 2-(phenethyloxy)tetrahydropyran 124

In order to investigate this effect, substrate **124**, which combines the axial positioning with the longer ether chain, was synthesised and subjected to the organism. A complex mixture of inseparable products (total yield 40 %) was obtained. This result suggested that the length of the linker was not the most important factor in determining selectivity.



124

Molecular modelling was used in an attempt to explain these differences in selectivity based on substrate structure. The molecular modelling was carried out using Macromodel[97] and the MM2 force field implemented therein.[98] The substrates were first constructed and energy minimised using a chloroform solvation method[99] and Monte Carlo simulations[100] were utilised to find the global energy minima rather than the local minimum resulting from the energy minimisation. Through this procedure, a number of the substrates, and their hydroxylated products, were studied. Examination of the results showed that the most populated lowest energy structures of each substrate had very similar conformations. Superimposition of these energy-minimised structures provided informative comparison.

Superimposition of the energy-minimised structures of substrates **112** (green) and **122** (red) about the hydroxylation sites (marked as balls) showed good overlap of the aromatic ring, exocyclic oxygen and heterocycle (figure 56).

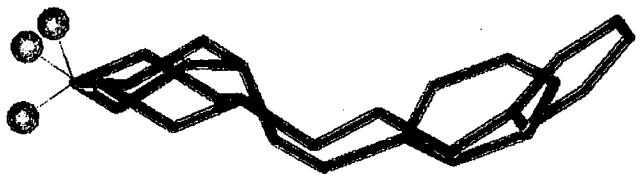


Figure 56: Superimposition of energy minimised substrate structures **112** (green) and **122** (red)

Examination of acetal **124** (blue), where the longer ether chain was situated axially showed that superimposition of the aromatic and acetal linkage left the heterocycle in a different orientation, where no hydrogen atoms sat in analogous positions to those hydroxylated in the other substrates (figure 57).

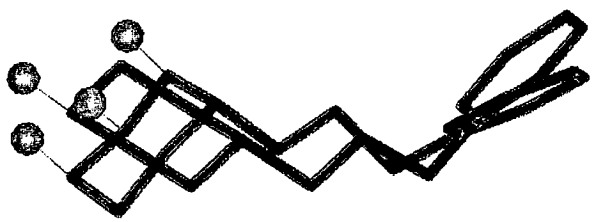


Figure 57: Superimposition of energy minimised substrate structures **124** (blue) and **122** (red)

This may demonstrate why substrates **112** and **122** were hydroxylated with similar regioselectivity, with the longer linker compensating for the difference in orientation at C-2 (which is axial **112** and equatorial in **122**). In contrast, acetal **124** was not hydroxylated with the same regioselectivity, presumably because the axial orientation of

the longer linker forced the heterocycle into a significantly different orientation with at least three possible hydroxylation sites possible.

Although such modelling studies can provide potential explanations for regioselectivity of hydroxylation based on substrate structure, it should be remembered that binding to the enzyme could cause the substrate to sit in a conformation that is significantly different to its energy-minimised conformation in the absence of the enzyme.

Yields of transformation

The product yields for the transformation of tetrahydropyranosyl derivatives by *Rhodococcus rhodochrous* were moderate as indicated in table 3. The yields reported are based on conversion of 100 mg with 5 g of wet cells and it should be noted that, in those cases where the reaction has been scaled up the yield was improved.

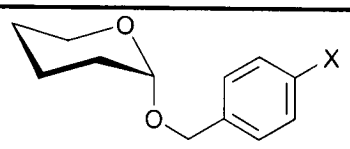
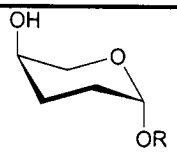
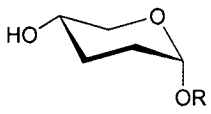
				
X =	Yield	[α_D]	Yield	[α_D]
Me (**113**)	9 % (**114**)	-21°	18 % (**115**)	+7°
Cl (**116**)	8 % (**117**)	-11°	12 % (**118**)	
NO_2 (**119**)	21 % (**120**)	+36°	18 % (**121**)	-44°

Table 3: Summary of hydroxylation of tetrahydropyran derivatives where R = CH_2ArX

The conversions reported were generally found to be highly reproducible with the exception of the *p*-chloro substituted case. In this case one or two products were isolated depending on the experiment and the *trans* product was favoured. It should be noted that the results reported for acetals **112** and **116** are different to preliminary results reported previously.[96]

The transformations reported were all carried out on racemic substrates and the optical activity of the products suggested a degree of stereoselectivity in the hydroxylation process. However, because the products described are not known compounds, the optical rotations give no indication of optical purity. Since less than 50 % of the mass balance is accounted for and no starting material was recovered, further breakdown of either substrate or product is possible. It was therefore not clear whether initial hydroxylation or further breakdown of the product was responsible for the observed stereoselectivity.

Interestingly when the aromatic side chain was altered as in ether **122**, the previously noted increase in regioselectivity was accompanied by an increased yield. Unfortunately the optical rotation of the product was negligible, suggesting that stereoselectivity was lost on insertion of the methylene unit.

Further investigation of the stereoselectivity of selected transformations is discussed in section 4.8.

Summary of biooxidation of tetrahydropyranosides

Examination of a range of closely related structures based on six-membered oxygen 2-alkyl heterocycles has demonstrated that *Rhodococcus rhodochrous* NCIMB 9703 can be a regioselective hydroxylation catalyst. It was observed that, irrespective of aromatic substituents, the enzyme has a preference for hydroxylation at C5, yielding mixtures of axial and equatorial substituted hydroxy products. Small structure modifications were seen to be potentially important in determining selectivity, leading, in some cases, to improved selectivity. The hydroxylations also showed a degree of stereoselectivity because racemic compounds were converted to optically active hydroxylated products.

4.7 Biooxidation of tetrahydrofuranosyl derivatives

The successful hydroxylation of the tetrahydropyran acetals prompted the investigation of the biotransformations of the corresponding tetrahydrofuran compounds. The results were seen to be largely analogous to those from the tetrahydropyran series. In the case of the tetrahydrofuran ring systems, the site of biohydroxylation was again determined by NMR studies (figure 58), although here it was not possible to obtain relative stereochemistry determinations from 1D NMR studies alone.

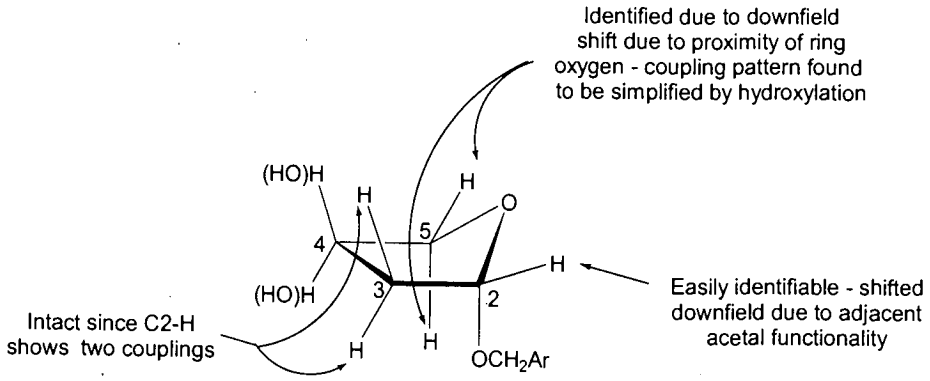
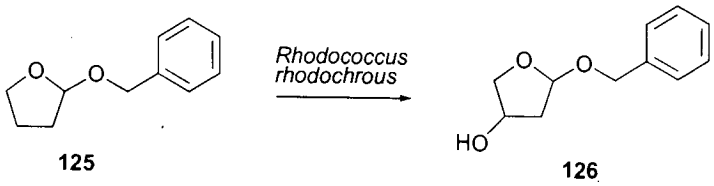


Figure 58: NMR analysis for determination of regiochemistry in products

Incubation of 2-benzyloxytetrahydrofuran 125

In the case of acetal **125**, a single product was isolated and identified as the C4 hydroxylated product **126** on the basis of its proton NMR spectrum. (figure 59)



125 126

Figure 59: Hydroxylation of 2-benzyloxytetrahydrofuran **125**

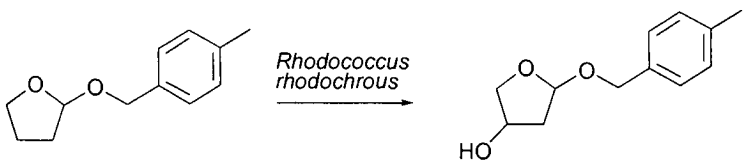
^{1}H-^{1}H COSY experiments (appendix 4) showed the characteristic C*H*OH signal coupling to both C5 protons and to the multiplets corresponding to the remaining ring protons which did not show any shift compared to the starting material. No coupling is seen between the C*H*OH and the C2-H signal. These findings can only be explained by substitution at the C4 position.

The relative stereochemistry of the two substituents on the ring cannot be identified from these simple experiments, since the bond angles on a five membered ring do not allow axial *versus* equatorial identifications from coupling constants. In common with the six membered analogues and the other compounds of the C4 ring series, the acetal existed in an axial orientation due to the anomeric effect.

Notably, the regioselectivity of this reaction was improved compared to the six membered analogue **112** that afforded a mixture of products. This may be due to the conformation of the five membered ring which presumably only allows one CH bond to be sufficiently close to the hydroxylating centre in the enzyme active site.

Incubation of 2-(4-methylbenzyloxy)tetrahydrofuran 127

Investigation of the hydroxylation of the methyl substituted analogue **127** showed that regioselectivity was not compromised and the analogous C4 hydroxylated product **128** was identified (figure 60).



127 **128**

Figure 60: Biohydroxylation of substituted tetrahydrofuran **127**

Incubation of 2-(4-chlorobenzyloxy)tetrahydrofuran 129

In the case of the *p*-chloro substituted 2-benzyloxytetrahydrofuran **129**, only a small amount of mixed hydroxylated furan derivative **130** was isolated along with around 30 % of *p*-chlorobenzoic acid **131** (figure 61).[96]

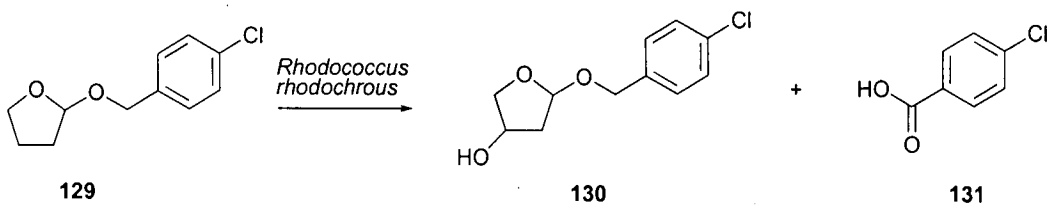
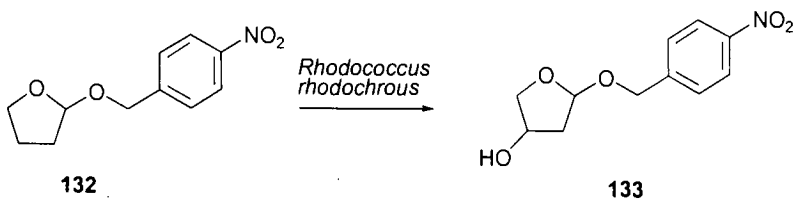


Figure 61: Hydroxylation of chloro substituted tetrahydrofuranoside **129**

The isolation of the benzoic acid derivative points toward benzylic hydroxylation followed by cleavage of the acetal. Degradation of alkyl-substituted aromatics by benzylic hydroxylation has been reported previously in whole cell systems[101] but to our knowledge, no systematic investigation of the effect of aromatic substituents has been undertaken. It is not clear at this stage why this particular substrate is degraded in this way. At first glance one might be inclined to assume that, since none of the other compounds in this series are noticeably degraded in this manner, the chlorine substituent on the aromatic must induce this degradation. However it should be remembered that the organism successfully hydroxylated tetrahydropyran analogue **116** with no benzoic acid derivative recovered. This suggests that the chloro substituent itself is not sufficient to induce such degradation.

Incubation of 2-(4-nitrobenzyloxy)tetrahydrofuran 132

The most interesting hydroxylation from this series was that of 2-(4-nitrobenzyloxy)-tetrahydrofuran **132** leading to one optically active product **133**. COSY, homodecoupling experiments and examination of the coupling patterns displayed in the proton NMR spectrum led to the identification of the product again hydroxylated in the C4 position (figure 62).

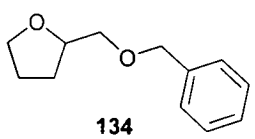


132 **133**

Figure 62: Regioselective transformation by *Rhodococcus rhodochrous*

Incubation of 2-(benzyloxymethyl)tetrahydrofuran 134

Following the success of the tetrahydrofuranoside **122**, the organism was challenged with the corresponding tetrahydrofuranoside **134**. In this case, the biotransformation produced only small amounts of a complex mixture of hydroxylated products that were not purified.

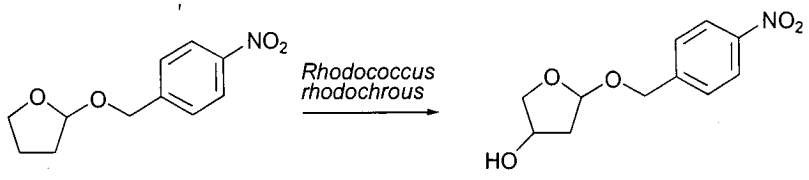


134

Yields of transformation

The product yields for the conversion of the tetrahydrofuranosides by *Rhodococcus rhodochrous* were moderate as indicated in Table 4. Again, the yields shown are based on conversion of 100 mg of starting material and were not optimised. However, it should be kept in mind that the oxidations appear to have occurred with concomitant kinetic resolution, and if this is so maximal yields would only be 50 %.

In common with the tetrahydropyran series, the hydroxylations reported were all carried out on racemic substrates. Again the products described are not known compounds, so the optical rotations give no indication of optical purity. A further investigation of the enantioselectivity of selected transformations is discussed later in section 4.8.



132 133

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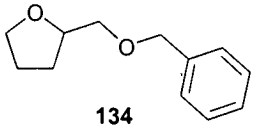

134

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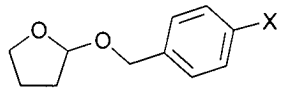
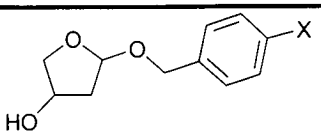
		
X =	Yield	$[\alpha_D]$
H (**125**)	15 % (**126**)	+45°
Me (**127**)	18 % (**128**)	+29°
NO_2 (**132**)	26 % (**133**)	+80°

Table 4: Summary of hydroxylation of tetrahydrofuranosides

Summary of biooxidation of tetrahydrofuranosides

Investigation of a number of closely related substrates based on saturated five-membered oxygen heterocycles demonstrated that *Rhodococcus rhodochrous* NCIMB 9703 can be a highly regioselective hydroxylation catalyst. The biocatalyst showed a strong preference for hydroxylation at C4, with only one product being isolated in most cases. Small structure modifications were important in determining regioselectivity, although the organism was less tolerant to changes in the tetrahydrofuranosides than in the six membered analogues. The insertion of a methylene group between the heterocycle and aromatic side chain significantly compromised the selectivity, resulting in small amounts of mixed products. *p*-Chloro substitution was not well tolerated in the five membered substrates leading to a mixture of benzylic and alkyl hydroxylation.

The fact that optical activity of products is observed suggests that the hydroxylations show a degree of enantioselectivity. In particular, incubation of 2-(4-nitrobenzyloxy)tetrahydrofuran **132** yielded product in higher yield and with higher optical rotation than the other reactions in this series and demonstrated the potential utility of this enzyme as a regio-, stereo- and enantioselective biohydroxylation catalyst.

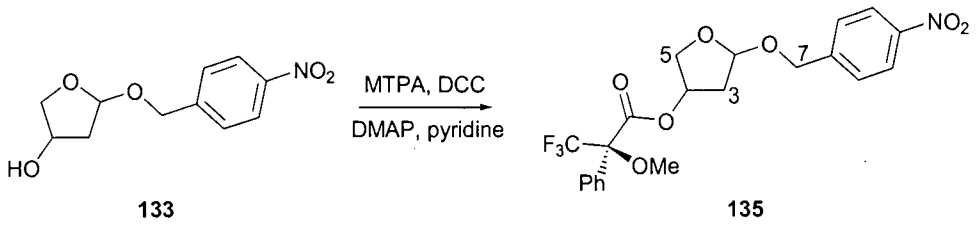
4.8 Stereoselectivity of hydroxylation

The nitro substituted products (**120**, **121** and **133**) from both series had particularly large values of optical rotation and were chosen as representative examples for further investigation.

2-(4-Nitrobenzyloxy)-4-hydroxytetrahydrofuran 133

We initially concentrated on the product **133** from tetrahydrofuranoside **132** and a number of derivatives were prepared. Many of the techniques for the determination of enantiomeric excess rely on the quantitative preparation of diastereomers from the enantiomer mixture with a chiral derivatising agent. Mosher's acid is one such derivatising agent and has long been the method of choice due to its stability to racemisation, substantial chemical shift differences between diastereomeric groups and the presence the trifluoromethyl group which allows the use of ^{19}F NMR spectroscopy for examination of the products.[102-104]

The α-methoxy-α-trifluoromethylphenylacetate (Mosher ester) **135** was prepared by reaction of alcohol **133** with *R*-α-methoxy-α-trifluorophenylacetic acid in the presence of dicyclohexylcarbodiimide (DCC) in dichloromethane. Examination of the ^{1}H and ^{19}F NMR spectra of the crude product suggested that a second product was also formed. This by-product was identified as the MTPA ester of 4-nitrobenzylalcohol **136**, envisaged to be formed *via* acid-catalysed acetal cleavage of **133** followed by esterification. When the reaction was carried out under basic conditions with DCC and 4-dimethylaminopyridine as acetylation catalyst, esterification was complete and no by-products were identified (figure 63). The product was not purified to avoid any resolution occurring.



133 135

Figure 63: Preparation of MTPA ester **135**

Only one of the possible four diastereomers is apparent by ^{1}H NMR spectroscopy of ester **135** with clear multiplets identified for C2, C3, C5 and C7 protons. Somewhat surprisingly, the methoxy signal was not a singlet as expected but a quartet resulting from long range fluorine-proton coupling ($^{4}J_{HF}$ 1 Hz). Examination of the simpler ^{19}F NMR spectrum (figure 64) however clearly shows two fluorine resonances from which a diastereomeric excess of 93% can be calculated.

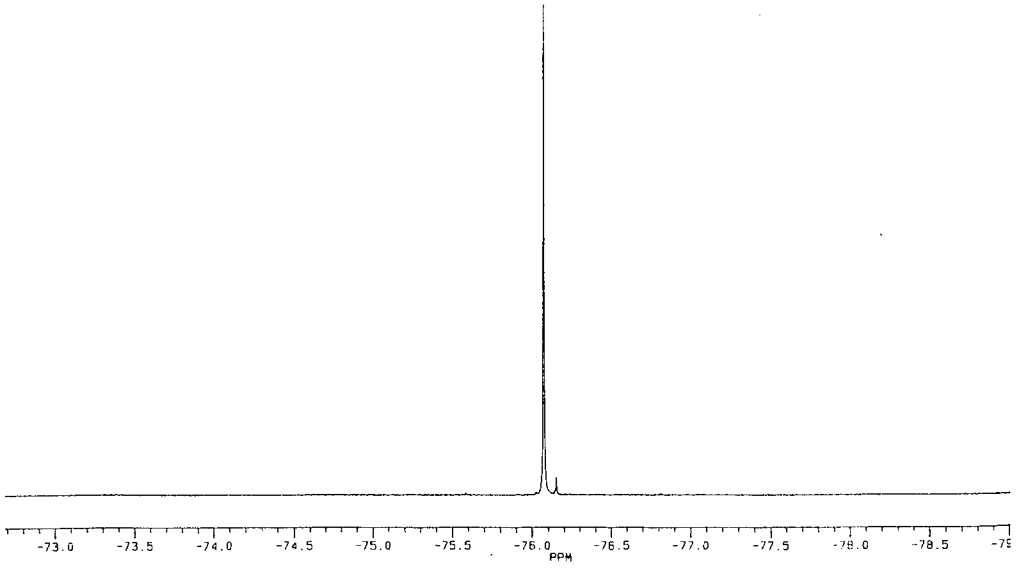
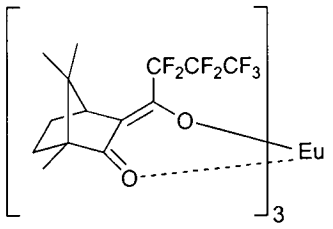


Figure 64: Fluorine NMR spectrum of ester **135**

Enantiomeric/diastereomeric excess can also be determined using lanthanide shift reagents to induce a chemical shift difference between diastereomeric groups.

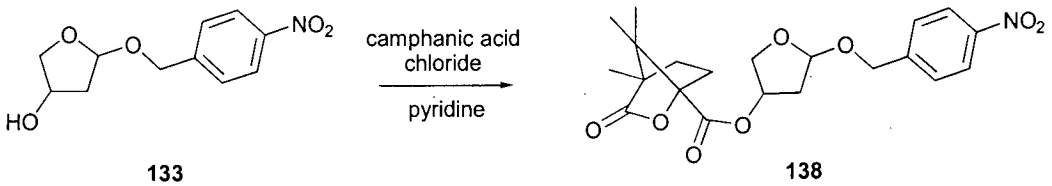
Combining the use of MTPA esters with lanthanide shift reagents has been reported to overcome problems of insufficient separation of signals in the ^{1}H NMR for analysis.[105] On the basis of the positive results reported, we attempted such analysis.



137

Unfortunately, stepwise addition of 5 mol % europium tris(3-heptafluoropropyl-hydroxymethylene)camphorate ($Eu(hfc)_3$) **137** to a sample of the MTPA ester **135** showed no separation of signals in the ^{1}H NMR before the increasing concentration of the lanthanide reagent caused significant line broadening.

Camphanic esters prepared from optically pure camphanic acid have previously been utilised for the preparation of crystalline material for determination of absolute stereochemistry.[106,107] The *S*-camphanic ester **138** was successfully prepared in 76% yield from reaction of alcohol **133** with *S*-camphanic acid chloride in the presence of pyridine (figure 65).



133 **138**

Figure 65: Preparation of camphanic ester **138**

Ester **138** appeared as only one diastereomer in the ^{1}H NMR as indicated by three sharp singlets for the methyl substituents on the camphanoate portion. Camphanamides have previously been studied for determination of enantiomeric excess by examination of the

diastereotopic methylene protons[108] and camphanic esters have been studied in conjunction with achiral shift reagents for determining enantiomeric purity.[109] These previous studies suggest that any significant second diastereomer should be visible in the NMR spectrum.

Crystals of ester **138** were obtained and re-crystallised from diethylether. The X-ray crystal structure showed the absolute stereochemistry of the tetrahydrofuran portion to be 2*R*, 4*R* as shown in figure 66. This *R,R* arrangement around the ring confirmed the tentative *trans* assignment of the relative ring stereochemistry based on NOESY experiments on the alcohol precursor.

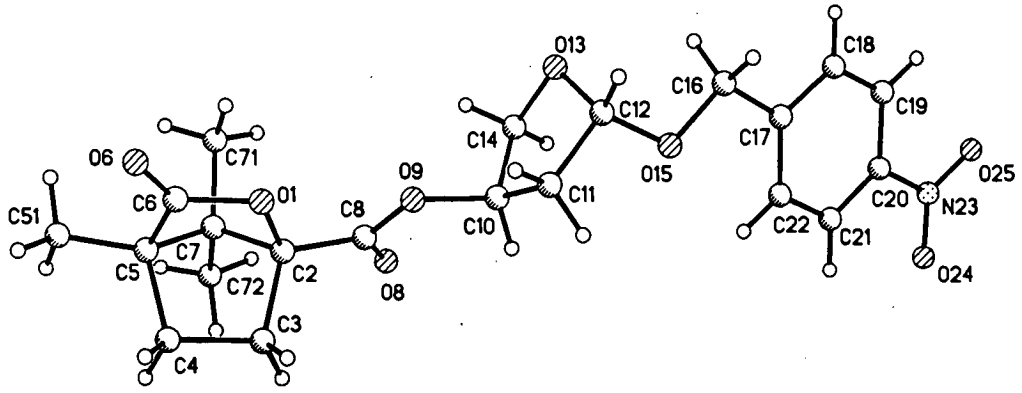


Figure 66: X-ray crystal structure of camphanic ester **138**

From the studies undertaken, it appears that biohydroxylation of 2-(4-nitrobenzyloxy)-tetrahydrofuran **132** by *Rhodococcus rhodochrous* NCIMB 9703 is a highly enantioselective process yielding the 2*R*, 4*R* product in circa 93 % enantiomeric excess.

We have thus identified a route to a chiral alcohol that is known to be a useful chiral intermediate. It is interesting to note that the open chain triol is currently accessed from *R*-malic acid in a simple one-pot procedure. The advantage of the biocatalytic route is that it allows for selective reactivity at both primary centres.

2-(4-nitrobenzyloxy)-5-hydroxytetrahydropyrans 120 and 121

Transformation of the 4-(nitrobenzyloxy)tetrahydropyran **119** yielded two C5 hydroxylated products which showed high optical rotations of +36° **120** and -44° **121** (figure 67).

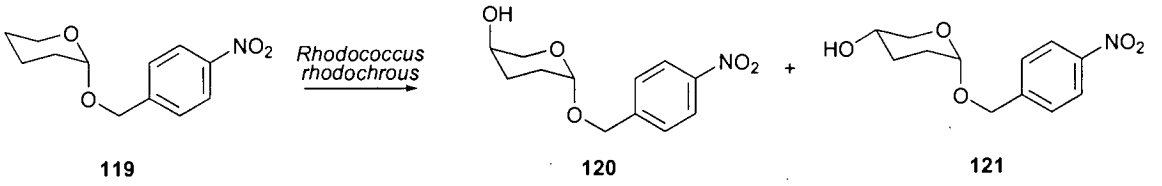


Figure 67: Hydroxylation of 4-nitro-substituted tetrahydropyran derivative **119**

Investigation of the enantioselectivity of hydroxylation was undertaken and esters prepared with the chiral derivatising agent MTPA and with camphanic acid with a view to accessing crystalline material.

cis-2-(4-Nitrobenzyloxy)-5-hydroxytetrahydropyran 121

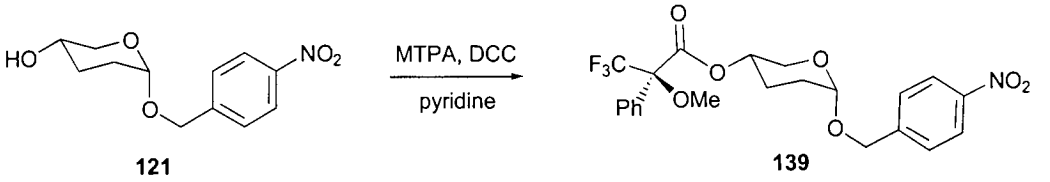


Figure 68: Preparation of MTPA ester

^{19}F NMR analysis of Mosher's ester **139** prepared from alcohol **121** (figure 68) showed only one peak suggesting an enantiomeric excess of greater than 95 %. Close examination of the ^{1}H NMR spectrum however cast doubt on this assignment (figure 69). Many of the signals in the spectrum were more complicated than they ought to be. In particular the C8-H signals, the diastereotopic benzylic protons, which should be a pair of AB doublets appeared as two overlapping pairs of AB doublets. This suggested that there was in fact a mixture of diastereomers present in the sample. Comparison of

integrals of the peaks suggested that the second signals accounted for almost 25 % of the sample. The signal for the methoxy group was a quartet with no indication of a further multiplet, which was surprising since the methoxy group would normally expected to show good chemical shift differences between diastereomers. Examination of the ^{13}C NMR agreed with the theory of a second diastereomer with a number of additional signals identified.

The conclusions drawn from the NMR studies of the MTPA ester **139** are contradictory which emphasises the problems in utilising any single method in isolation. It is possible that the ^{19}F signals for more than one of the possible diastereomers simply overlap and that examination of the proton NMR spectrum is more reliable. The only method of conclusively measuring the enantiomeric excess and determining the stereochemistry of the product would be comparison with authentic samples. Unfortunately, preparation of authentic samples of all of the possible diastereomers was outwith the scope of this project.

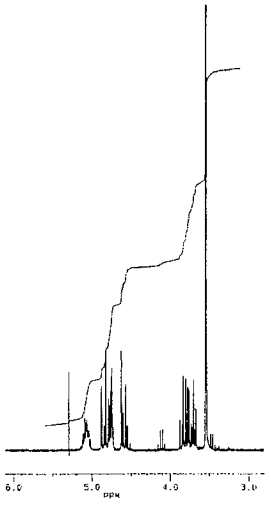


Figure 69: Proton NMR spectrum of ester **139** in the 3 –6 ppm range

The preparation of a camphanic ester was helpful for investigation of the five membered analogue and was therefore undertaken for alcohol **121** yielding camphanic ester **140** (figure 70).

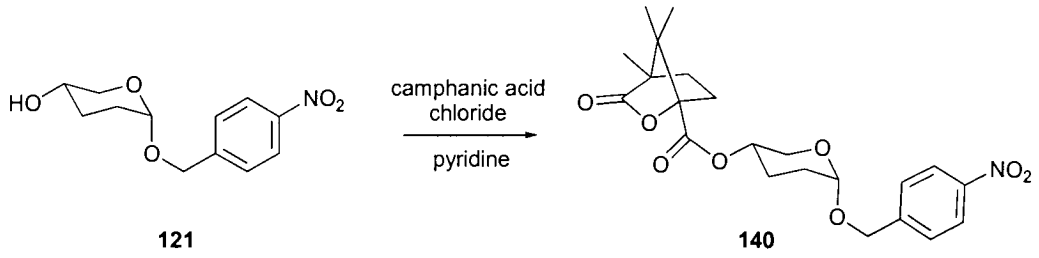


Figure 70: Preparation of camphanic ester **140**

Contrary to the results observed for the MTPA ester **139**, both the ^{1}H (figure 71) and ^{13}C NMR spectra of the camphanic ester were easily assigned and showed no indication of more than one diastereomer of **140**. Camphanic ester **140** has not, as yet, produced crystalline material.

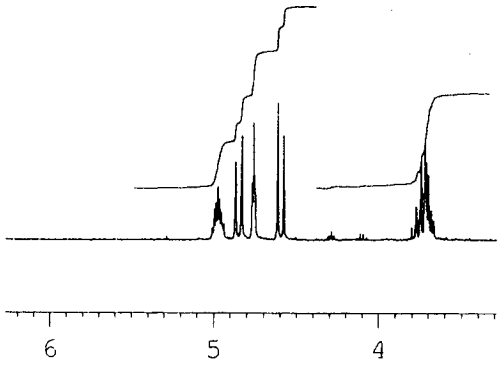


Figure 71: Proton NMR expansion of ester **140**

*trans-2-(4-Nitrobenzyloxy)-5-hydroxytetrahydropyran **120***

MTPA ester **141** (prepared from alcohol **120**) showed two resonances in the ^{19}F NMR of approximately equal peak area (figure 72). If the two peaks present in the fluorine NMR spectrum were due to diastereomers, this would give a diastereomeric excess of only 7 %. However, since both the ester and its alcohol precursor had large optical rotations, it seemed unlikely that the ester was a near equal mixture of diastereomers.

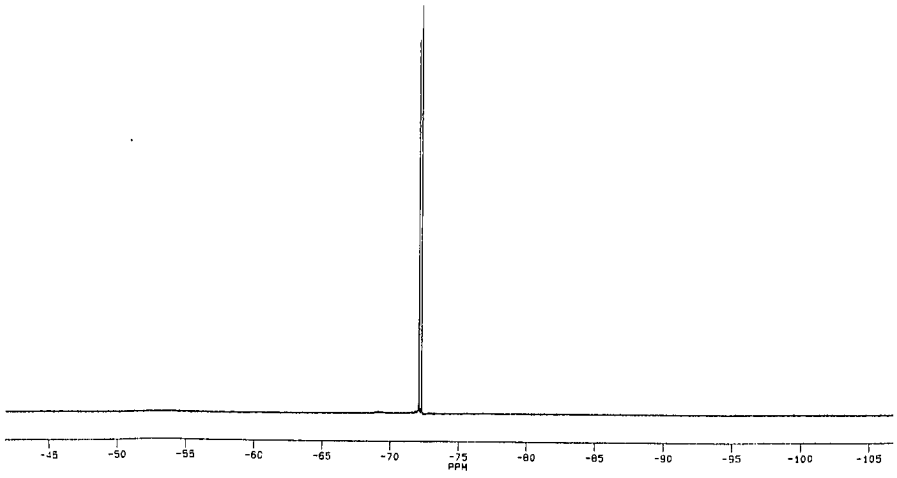


Figure 72: Fluorine NMR spectrum of ester **141**

Examination of the ^{1}H NMR spectrum clearly showed a mixture of compounds in the sample, in particular the methoxy and C6-H*eq* and C6-H*ax* signals clearly showed multiplets for different molecules and other signals in the spectrum were much broader than would be expected. Carbon-13 NMR spectroscopy also showed the existence of more than one compound with secondary peaks identified at similar chemical shift to the main peak.

We suggest that the product is not a mixture of diastereomers but that, on formation of the bulky ester from the axial hydroxyl group, the diaxial ring strain is increased sufficiently that the energy of the system can overcome the anomeric effect and cause the ring to flip to produce the more favourable diequatorial arrangement around the tetrahydropyran ring as shown in figure 73.

Figure 73: Possible ring flipping to produce a mixture of conformers

This effect would not be expected for ester **139** where the bulky ester group already exists in the equatorial position on the ring. NMR experiments at elevated temperatures (323 and 343 K) were carried out in order to investigate whether there was an equilibrium effect but at the temperatures which we could raise the sample to, no change was noted and these experiments provided no further evidence to explain this result.

In common with the other alcohols under study, the camphanate derivative **142** was also prepared by reaction with camphanic acid chloride (figure 74) and the resultant ester **142** analysed.

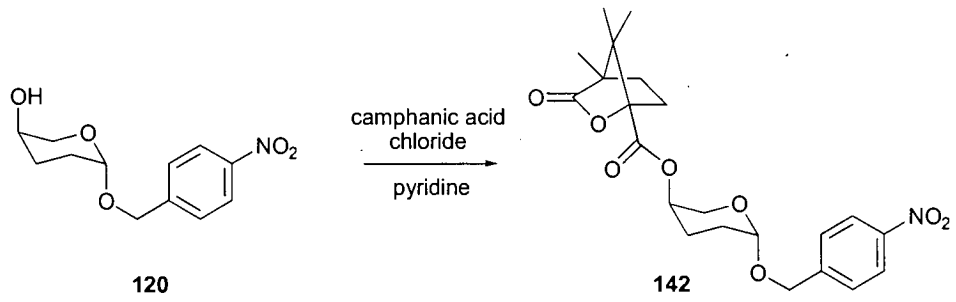


Figure 74: Preparation of camphanic ester **142**

Carbon-13 NMR spectroscopy showed a number of additional peaks and ^{1}H NMR spectrum clearly showed that the methyl signals were not singlets. Crystallisation has as yet been unsuccessful, further suggesting a mixture of compounds.

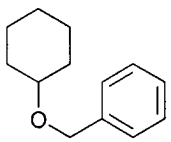
4.9 Biooxidations of carbocyclic ethers

After successful investigation of the five and six membered oxygen heterocycles showed the potential of *Rhodococcus rhodochrous* NCIMB 9703 as a regio and enantioselective hydroxylation catalyst, the obvious substrates to investigate next were the corresponding carbocycles. Not only would this yield information as to the importance of the cyclic oxygen for selectivity but it is also envisaged that the hydroxylated carbocyclic

compounds could be synthetically useful. Thus carbocycles of similar structure were prepared and examined as hydroxylation substrates for the organism. Unfortunately, the results from this series were less promising than the corresponding heterocycles with the incubations leading to mixtures of products.

Incubation of benzyloxycyclohexane 143

Rhodococcus rhodochrous NCIMB 9703 converted the simple benzyloxycyclohexane **143** to two inseparable hydroxylated products in good combined yield (35 %). Acetylation of the product mixture failed to improve the separation of the two products. Based on the results from the oxygen heterocycles and those reported previously,[96] it is thought likely that these were the C4 hydroxylated compounds (C4 is analogous to the C5 position in the tetrahydropyranosides).



143

Incubation of benzyloxycyclohexene derivatives

The organism was challenged with cyclohexenol derivatives with the aim of accessing intermediates which could be further functionalised, however these transformations proved to be rather unsuccessful. 4-Benzyloxycyclohex-1,2-ene **144** was found to yield the corresponding epoxide **145** in good yield (figure 75). Unfortunately, the epoxide showed negligible optical activity suggesting little enantiodifferentiation.

144 **145**

Figure 75: Production of epoxide by *Rhodococcus rhodochrous*

Three products were isolated on incubation of 3-benzyloxycyclohex-1,2-ene **146** (figure 76). The 3-ketone derivative **147** was isolated, presumably due to hydroxylation of an alkene carbon and subsequent rearrangement.[125,126] Two saturated alcohols **148** and **149** were also identified in the product mixture suggesting that reduction of the double bond was taking place in addition to the expected hydroxylation.

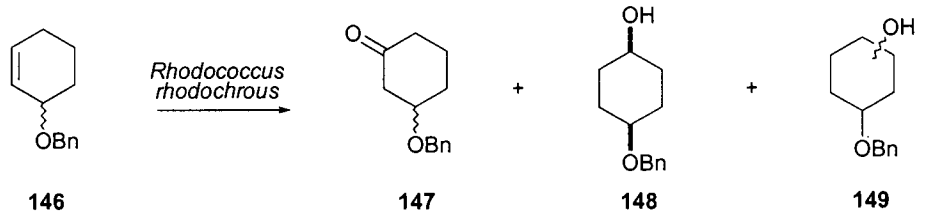


Figure 76: Mixture of products isolated from incubation of cyclohexenol derivative **146**

It should be noted that the results reported for alkenes **144** and **146** do not agree with preliminary results reported previously.[96]

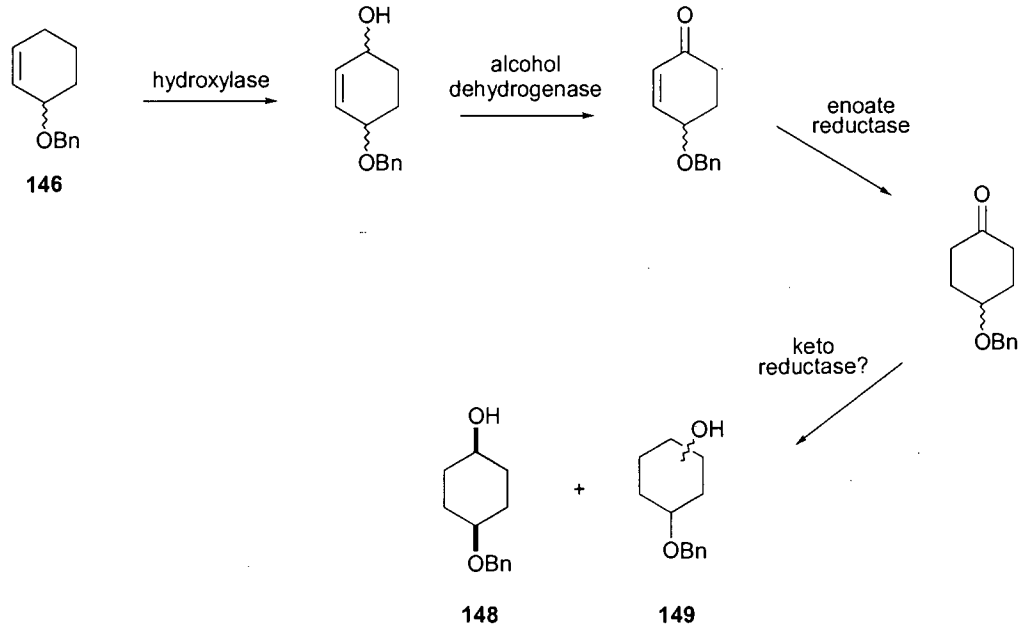


Figure 77: Proposed route to saturated products

Allylic alcohols are possible products from initial hydroxylation of alkenes and are known to be substrates for enoate reductases, which carry out the reduction of activated double bonds[110] and may provide a route to the saturated alcohols isolated (figure 77). This reduction of the double bond in addition to the desired hydroxylation emphasises the potential problems in utilising whole cell systems since side reactions are often unpredictable.

Summary of biooxidation of carbocyclic ethers

Investigation of the carbocyclic compounds showed less selective conversion than the analogous oxygen heterocycles. Most notable is that the anomeric effect cannot operate for these compounds so that the spatial orientation of the aromatic side-chain is different. This factor has already been shown to have an important effect on the selectivity of hydroxylation.

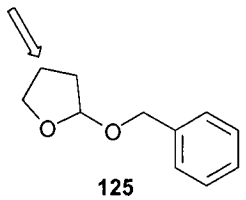
Investigation of the unsaturated carbocycles **144** and **146** showed other competing reactions that were not relevant for the saturated substrates. Interestingly no benzylic hydroxylation, the only side reaction previously noted with the organism, was observed. The isolated products were found to have negligible optical rotations suggesting minimal enantioselectivity. This result, in conjunction with our results from the tetrahydropyranosides, points toward an important role for the orientation of the aromatic side-chain in determining the enantioselectivity, with axial positioning leading to improved enantioselectivity.

4.10 Biooxidations of nitrogen heterocycles

Enzymatic hydroxylation of protected nitrogen heterocycles have previously been studied and found to be an important route to optically active hydroxylated nitrogen heterocycles. A recent report by Witholt and co-workers described the production of optically active *N*-benzyl-3-hydroxypyrrolidine by *Pseudomonas oleovorans*.[111]

Optically active hydroxypyrrolidines are important intermediates in the synthesis of a number of biologically important molecules and although a number of synthetic routes exist, each has drawbacks such as expensive chiral starting materials or low yielding resolution steps.

Due to the structural similarities to our previously studied substrates (figure 78), we prepared *N*-benzylpyrrolidine for testing with *Rhodococcus rhodochrous*. It was found that incubation of the substrate with resting cell cultures of the organism resulted in no hydroxylation and the starting material was recovered unchanged.



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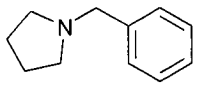


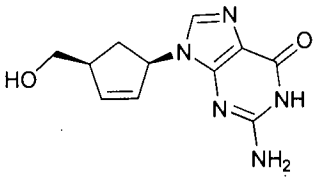
150

Figure 78 :Comparison of two compounds under study

Since this transformation was unsuccessful, no further nitrogen heterocycles were tested as substrates for the biocatalyst although further investigation of this substrate type, perhaps employing a longer aromatic chain, could be interesting.

4.11 Biooxidations of carbocyclic nucleoside precursor

Carbocyclic nucleosides show biological activity particularly as antiviral and antitumour agents and possess greater metabolic stability to phosphorylase enzymes that cleave the linkage between the heterocyclic base and sugar molecule in the corresponding nucleosides. Two carbocyclic analogues, Carbovir **43** and Abacavir **151**, are of particular interest due to their anti-HIV activity. Carbovir[112] is already in clinical use and Abacavir[113] has recently completed clinical trials.



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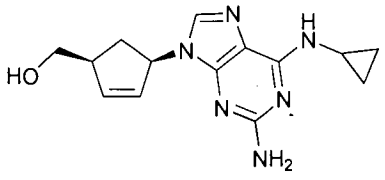
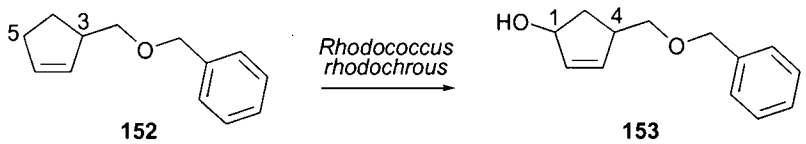


151

There are many routes to carbocyclic nucleosides, some of which require initial formation of an appropriately functionalised carbocycle followed by coupling of the required base. This method requires enantiomerically pure cyclopentane and cyclopentene units and biocatalytic routes have been employed in their preparation.

We were aware that the carbocyclic analogues of the compounds under study were structurally related to such carbocyclic nucleoside precursors. We therefore sought to investigate whether the substrate range of the enantioselective hydroxylating activity could be extended to include compounds such as **152**.

Incubation of ether **152** resulted in the isolation and identification of one hydroxylated product **153** in around 20 % yield. The product was identified as the C5 hydroxylated product (figure 79) on consideration of a number of factors. Examination of proton and carbon NMR spectrum showed that the alkene portion was intact in the product molecule. C3 was also intact but its complicated coupling pattern did not allow determination of substitution at C4. Importantly, the alkene signals were noticeably simplified in the product suggesting that hydroxylation took place at C5.



152 153

Figure 79: Hydroxylation of ether **152** by *Rhodococcus rhodochrous*

Two-dimensional NOESY experiments were carried out in an attempt to determine the relative stereochemistry around the ring. The two important signals for investigation of the relative stereochemistry around the ring are those for H1 and H4, the protons adjacent to the ring substituents. As indicated in figure 80, H1 shows nOe crosspeaks to the opposite alkene proton and to the opposite C4 proton to H4 and the two signals under study do not show an nOe to each other. This analysis of the crosspeaks in the NOESY spectrum suggests a *trans* arrangement around the ring.

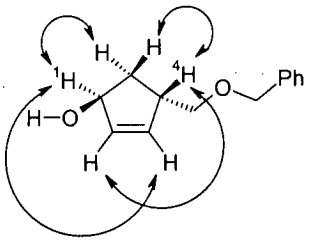
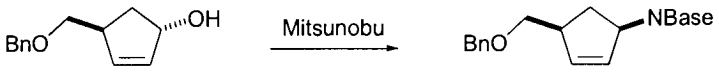


Figure 80: nOe crosspeaks indicated by arrows

It was envisaged that this *trans* hydroxylated product could be easily converted to the protected carbocyclic nucleoside by Mitsunobu reaction at the introduced alcohol (figure 81).



153 **154**

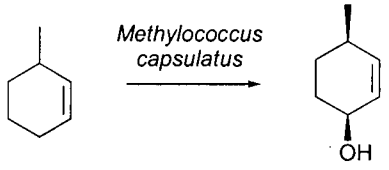
Figure 81: Proposed route to carbocyclic nucleoside derivatives **154**

The product was seen to have a significant optical rotation triggering an investigation into the enantioselectivity of the biohydroxylation. Since little product was available in this initial phase of the project only the camphanic ester was prepared. Proton and carbon NMR analysis showed more peaks than would be expected and thus suggested that the reaction might not be 100 % stereoselective. Crystallisation studies have, as yet,

been unsuccessful. Clearly further investigation of the enantioselectivity of hydroxylation is necessary.

Work is currently underway within the group to determine the enantioselectivity of transformation, confirm the relative stereochemistry and utilise the biotransformation product as a chiral intermediate in the synthesis of carbocyclic nucleosides.

Allylic hydroxylation is not uncommon in microorganisms and although many of the reports involve rigid steroid molecules, there are also examples of extremely selective hydroxylation in non-rigid, non-activated substrates (figure 82).[114]



155 156

Figure 82: Microbial allylic hydroxylation

4.12 Nature of the hydroxylating enzyme

Rhodococcal hydroxylases have been reported previously and it is clear that at least one is functioning in the organism under study. *Rhodococcus erythropolis* DSM 6138 has recently been shown by Gotor and co-workers to contain a molybdate dependent oxygenase that is responsible for the hydroxylation of, for example, pyrimidine heterocycles.[27] Cytochrome P-450 monooxygenases are also widespread in *Rhodococcus* species: a cytochrome P-450 responsible for the 6-*endo*-hydroxylation of camphor has recently been identified and purified from *Rhodococcus sp.* NCIMB 9784.[68] As part of our investigation into the use of cytochrome P-450 monooxygenases in biocatalysis, we were interested to examine the nature of the hydroxylase.

Alkane hydroxylators have previously been shown to hydroxylate non-natural substrates. Another octane utilising enzyme, *Pseudomonas oleovorans* is known to contain a cytoplasmic membrane monooxygenase, which carries out the ω-hydroxylation of octane. A recent publication by Witholt *et al*[111] has shown the utility of the alkane hydroxylase system by preparing optically active hydroxylated *N*-benzylpyrrolidine (as described in section 4.8).

Initial literature reports suggested that terminal hydroxylation of *n*-octane by *Rhodococcus rhodochrous* NCIMB 9703 is catalysed by an inducible cytochrome P-450.[48,65] In this work we hoped to utilise this cytochrome P-450 as a biohydroxylation catalyst. The reactions reported here were carried out using cells grown on octane as the sole carbon source, but cells grown on sodium pyruvate were also tested in an attempt to establish the nature of the hydroxylase. Comparable yields and optical activities of products were observed utilising both octane and pyruvate grown cells, suggesting that these reactions do not utilise the octane-hydroxylating enzyme, or that there is an unknown limiting factor in operation. Such a limiting factor could render the expression level of the protein insignificant and could be NADH supply, substrate diffusion or oxygen transfer into the cell.

Inhibition studies using 1-aminobenzotriazole and metyrapone showed that hydroxylation was inhibited by both of these compounds. Because these cytochrome P-450 inhibitors inhibit the enzyme by different mechanisms,[27,89] these studies were taken to be evidence that a cytochrome P-450 system was operating in these transformations.

Thus far, these studies have yielded only limited information about the hydroxylase under study but it is clear that the hydroxylations reported are cytochrome P-450 catalysed. As in chapter 3, it is unclear whether the hydroxylase operating is the induced cytochrome P-450 or another constitutively expressed enzyme.

4.13 Optimisation of transformations

Although some studies have been undertaken to improve the biotransformation, there are many avenues for optimisation of the whole cell system as a hydroxylation catalyst. The effect of substrate concentration has been investigated and the optimum concentration found to be around 0.5 gL^{-1}, with higher concentrations of up to 0.6 and 0.7 gL^{-1} tolerated for some substrates. The exact concentration tolerance seems to be substrate dependent and would require further investigation for larger scale preparations.

Another extremely important variable is cell concentration. In some studies, the authors report extensive investigation to optimise this parameter.[111] In our case, the growth of the organism was the slowest stage in the testing of substrates and we therefore sought to minimise the cell mass used for each reaction. We found that the use of 5 g wet cell mass for transformation of 100 mg substrate (200 mL volume) was the minimum cell mass that did not increase the required time unnecessarily.

The time of transformation is an important factor, both in terms of practicality and selectivity. In the hydroxylations described, optically active products were isolated in yields of up to 40 %. The mass balance of the reaction is thought to be lost through degradation pathways from the substrate or the hydroxylated product. The enantiomeric excess of the isolated product is likely to be dependent on a number of factors including further degradation of the desired product. In future, periodic sampling of aliquots should provide data to optimise this.

Co-metabolism of a co-substrate such as glucose could also be advantageous since this provides energy for the reaction and the recycling of co-factors that could increase the rate sufficiently to impact on the yield or selectivity of hydroxylation.

Work is currently underway on the optimisation of conditions for the preparation of carbocyclic nucleoside precursors.

4.14 Conclusions

This study has established that *Rhodococcus rhodochrous* NCIMB 9703, an organism not previously reported for biotransformations of non-natural substrates, can be used to regioselectively and enantioselectively hydroxylate a range of oxygen heterocycles. It was also noted that the regio- and enantioselectivity of reaction was significantly altered by changes to the *p*-substituent on the benzyloxy group or by extension of the ether linkage. It is envisaged that this biocatalyst could be used to produce hydroxylated, polyfunctionalised chiral intermediates for use in asymmetric synthesis.

2-Benzyloxytetrahydropyrans were substrates for resting cell suspensions of the bacteria yielding predominantly 5-hydroxylated products in combined yields of up to 40 %. Corresponding tetrahydrofuranosides were also hydroxylated, mainly to 4-hydroxy compounds in yields of up to 26 %. Most interestingly, 2-(4-nitrobenzyloxy)-tetrahydrofuran **125** was transformed exclusively to the 4-hydroxylated product **130** with an optical rotation of +80° in 93 % enantiomeric excess and the 2-(4-nitrobenzyloxy)-tetrahydropyran **115** was hydroxylated exclusively in the 5-position giving two products with optical rotation +36° and -44°.

The transformation of a carbocyclic nucleoside precursor **152** was investigated with the desired *trans* product identified as the only product. The hydroxylation was found to be enantioselective in addition to its high regiospecificity. This particularly positive result is currently under further study within the group.

5 Experimental

5.1 Apparatus and chemicals

All chemicals were purchased from the Aldrich Chemical Company, Poole, Dorset U.K. or Acros Organics, Loughborough, U.K.. ^{1}H and ^{13}C NMR analysis was performed on Bruker AC250 (250 MHz and 63MHz respectively) and WH-360 (360 MHz and 90 MHz) spectrometers. ^{19}F NMR analysis was performed on a Bruker AC250 spectrometer at 235 MHz. Unless otherwise specified, spectra were recorded in deuterochloroform at ambient temperature. Chemical shifts are quoted in parts per million (ppm) and were referenced internally (^{1}H, $CHCl_3$, 7.27 ppm; $^{13}CHCl_3$, 77.0 ppm). Carbon multiplicity was established by DEPT (distortionless enhancement by polarisation transfer).

Infra-red spectra were recorded on a Perkin-Elmer FT-IR spectrometer (Paragon 1000) as thin films with frequencies (ν) measured in wavenumbers (cm^{-1}). Mass spectra were run using electron impact (EI) on Finnigan 4500 or 4600 instruments. Thin layer chromatography was performed on glass sheets coated with silica gel Merck 60F-254 (0.24 mm, Art. 5715). Components were detected by UV (254 nm) and visualised by treating the plate with ammonium molybdate solution and heating. Wet flash chromatography was carried out on silica gel (Merck 9385, Kieselgel 60).

Optical rotations [α_D] were measured on an Optical Activity AA-1000 polarimeter with a cell path length of 1 dm at a temperature of 25 °C and concentrations (c) quoted in g/100 mL in methanol (sodium 589 nm detection). Unless otherwise stated, the compounds reported have been prepared in racemic form.

Gas chromatography (GC) was carried out using a Hewlett-Packard 6890 series GC system with flame ionisation detection or mass selective detection (HP 5973) employing a HP-5MS 5% phenyl methyl siloxane capillary column (30.0m x 320μm x 0.25μm).

The HPLC analysis was carried out on a Waters 486 system employing Millennium software using a Sphericlone® 5 μ ODS-2, 250 x 4.6 mm column (Phenomenex). Preparative scale HPLC was carried out on an equivalent column with an internal diameter of 10.0 mm at a flow-rate of 4.5 mL/min.

5.2 *Beauveria bassiana* ATCC 7159

5.2.1 Maintenance and growth of microorganism

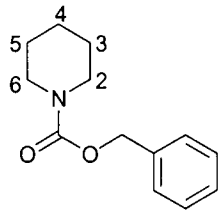
Beauveria bassiana ATCC 7159 was obtained from the American Type Culture Collection, U.S.A.. The organism was maintained on malt extract agar plates at room temperature and these were subcultured at regular intervals. A loop of fungus was used to inoculate 60 mL of sterile medium containing 7.5 g L^{-1} corn steep solids and 10 g L^{-1} glucose in distilled water adjusted to pH 4.85 in a 250 mL Erlenmeyer flask. After three days growth on an orbital shaker at 200 r.p.m. at 25° C, the culture was used to inoculate 600 mL of the same medium in a 2 L Erlenmeyer flask. This culture was again grown for three days.

5.2.2 General biotransformation procedure

A solution of 66 mg substrate in 1 mL ethanol was prepared and added to a three day old 660 mL culture of *Beauveria bassiana* ATCC 7159. After a further three days incubation as above, the fungal cells were removed from the broth by centrifugation and the supernatant extracted into ethyl acetate. Purification of metabolites was routinely carried out using wet flash chromatography with petroleum ether/ethyl acetate gradients as eluent.

5.2.3 Results of biotransformations

Typical numbering scheme used for substituted piperidines under study



Biohydroxylation of N-carboxybenzylpiperidine 57[74] produced hydroxylated product **58** that was isolated as a colourless oil (100 mg, 33 %). This piperidinol has been reported and characterised previously.[115,116]

Biohydroxylation of N-carboxybenzyl-4-methylpiperidine 62[74]

N-carboxybenzyl-4-methyl-4-hydroxy-piperidine **63** was isolated as a pale yellow solid (130 mg, 45 %); mp 76 °C; δ_H (250 MHz) 1.24 (3H, s, CH_3); 1.45 - 1.56 (5H, m, C(3)H_2, C(5)H_2 & C(4)OH), 3.23 - 3.34 (2H, dt, C(2)H*ax* & C(6)H*ax*, *J* 7 & 13), 3.80 (2H, br. d, C(2)H*eq* & C(6)H*eq*, *J* 13), 5.11 (2H, s, CH_2Ph), 7.25 - 7.36 (5H, m, aromatic); δ_C (63 MHz) 30.0 (q, CH_3), 38.2 (t, C3 & C5), 40.2 (t, C2 & C6), 66.9 (t, CH_2Ph), 67.7 (s, C4), 127.7 (d, aromatic), 127.8 (d, aromatic), 128.3 (d, aromatic), 136.7 (s), 155.1 (s); *m/z* (EI) required 249.13649, found 249.13637; 249 (44 %, M^+), 158 (14, M - $PhCH_2$), 142 (16, M - $PhCH_2O$), 91 (100, $PhCH_2$).

Biohydroxylation of N-carboxybenzyl-3-methylpiperidine 67

Three products were isolated from this biohydroxylation, two of which were found to be difficult to separate and were thus separated by subjecting the mixture to acetylation conditions (10 mol equivalents acetic anhydride, 5 mol equivalents pyridine and catalytic dimethylaminopyridine) under which conditions one of the products was

acetylated **69** and one was left untouched **70**. The products were then separated by preparative HPLC

N-carboxybenzyl-trans-3-methyl-4-hydroxypiperidine **68** was isolated as a colourless oil (10 mg, 3 %); δ_H (360 MHz) 0.99 (3H, d, CH_3, *J* 6.5), 1.43 - 1.59 (3H, br. m) & 1.91 (1H, br. dd, *J* 3.& 13) (C(3)H, C(4)OH or C(5)H_2), 2.53 (1H, br. s, C(2)H*ax* or C(6)H*ax*), 2.90 (1H, ddd, C(4)H, *J* 3, 12 & 14), 3.30 (1H, br. m, C(2)H*ax* or C(6)H*ax*), 4.05 - 4.13 (2H, br. m, C(2)H*eq* & C(6)H*eq*), 5.11 (1H, s, CH_AH_BPh), 5.12 (1H, s, CH_AH_BPh), 7.28 - 7.36 (5H, m, aromatic); *m/z* (EI) required 249.13649, found 249.13616; 249 (8 %, M^+), 158 (11, M - $PhCH_2$), 142 (6, M - $PhCH_2O$), 91 (100, $PhCH_2$).

N-carboxybenzyl-cis-3-methyl-4-hydroxypiperidine was isolated as its acetylated derivative **69a** as a colourless oil (5 mg); δ_H (360 MHz): 0.88 (3H, d, CH_3, *J* 7). 1.67 - 1.93 (3H, br. m, C(3)H & C(5)H_2), 2.07 (3H, s, acetyl CH_3), 3.00 - 3.15 (1H, br. m, C(2)H*ax* or C(6)H*ax*), 3.25 - 3.28 (1H, br. m, C(2)H*ax* or C(6)H*ax*), 3.70 (1H, br. s, C(2)H*eq*), 3.75 (1H, ddd, C(6)H*eq*, *J* 4.5, 4.5 & 13.5), 5.01 (1H, ddd, C(4)H*eq*, *J* 3, 3 & 5), 5.12 (2H, d, CH_2Ph, *J* 2), 7.28 - 7.39 (5H, m, aromatic); *m/z* (EI) required 291.14706, found 291.14768; 291 (3 %. M^+), 200 (11, M - $PhCH_2$), 91 (100, $PhCH_2$).

N-carboxybenzyl-3-methyl-3-hydroxypiperidine **70** was isolated as a colourless oil (10 mg, 3 %); δ_H (360 MHz) 1.21 (3H, s, CH_3), 1.49 - 1.79 (5H, m, C(3)OH, C(4)H_2 & C(5)H_2). 2.96 - 3.03 (2H, br. m, C(2)H*ax* & C(6)H*ax*), 3.68 (1H, br.s, C(2)H*eq*), 3.84 (1H, ddd, $C(6)H_{eq}$, *J* 4.5, 4.5 & 13), 5.13 (2H, s, CH_2Ph), 7.28 - 7.35 (5H, m, aromatic); *m/z* (EI) required 249.13649, found 249.13725; 249 (5 %, M^+), 114 (26, M - $PhCH_2OCO$), 91 (100, $PhCH_2$).

Biohydroxylation of N-carboxybenzyl-2-methylpiperidine 71

N-carboxybenzyl-cis-2-methyl-4-hydroxy-piperidine **72** was isolated as a colourless oil (20 mg, 7 %); δ_H (250 MHz) 1.35 (3H, d, CH_3, *J* 7), 1.46 - 1.91 (5H, m, C(3)H_2, C(4)OH & C(5)H_2), 3.34 (1H, ddd, C(6)H*ax*, *J* 3.5 & 12.5), 3.90 (1H, ddd, C(6)H*eq*, *J* 3.5, 4 & 12.5), 4.16 (1H, q, C(4)H, *J* 3), 4.31 - 4.42 (1H, m, C(2)H*eq*), 5.12 (2H, s, CH_2Ph), 7.25 - 7.36 (5H, m, aromatic); δ_C (90 MHz) 19.1 (q, CH_3), 32.2, 33.5 (t, C3 & C5), 36.4 (t, C6), 45.7 (d, C2), 64.7 (d, C4), 66.8 (t, CH_2Ph), 127.7 (d, aromatic), 127.8 (d, aromatic), 128.4 (d, aromatic), 136.9 (s), 155.2 (s); *m/z* (EI) required 249.13649, found 249.136337, (FAB) 234 (4 %, M - CH_3), 158 (15, M - $PhCH_2$), 142 (31, M - $PhCH_2O$), 114 (2, M - $PhCH_2CO_2$), 91 (100, $PhCH_2$).

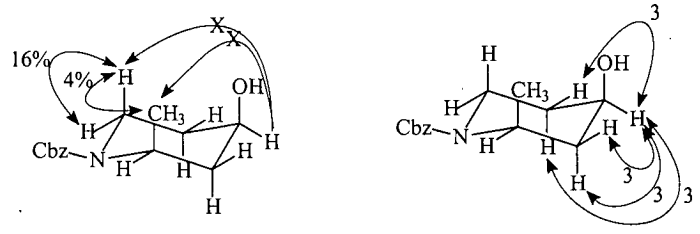


Figure 83: nOe enhancements and coupling constants used in assigning relative stereochemistry in product **72**

N-carboxybenzyl-cis-2-methyl-3-hydroxy-piperidine **73** was isolated as a colourless oil (20 mg, 7 %); δ_H (360 MHz) 1.12 (3H, d, CH_3, *J* 7), 1.43 - 1.78 (5H, m, C(3)OH, C(4)H_2 & C(5)H_2), 2.79 (1H, ddd, C(6)H*ax*, *J* 3, 13 & 13.5), 3.77 (1H, m, C(3)H), 3.95 (1H, br d, C(6)H*eq*, *J* 12.5), 4.51 (1H, q, C(2)H*eq*, *J* 6.5), 5.12 (2H, s, CH_2Ph), 7.25 - 7.38 (5H, m, aromatic); δ_C (90 MHz) 9.2 (q, CH_3), 24.0 (t, C4 or C5), 27.1, (t, C4 or C5), 37.6 (t, C6), 51.0 (d, C2), 67.0 (t, CH_2Ph), 69.0 (d, C3), 127.7 (d, aromatic), 127.9 (d, aromatic), 128.4 (d, aromatic), 136.7 (s), 155.3 (s); *m/z* (EI) required 249.13649, found 249.13637, (APCi) 250 (100 %, MH^+), 158 (7, M - $PhCH_2$).

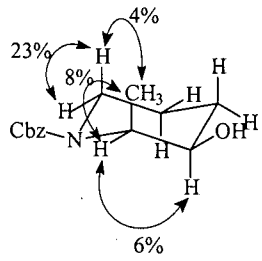


Figure 84: nOe enhancements used in assigning the relative stereochemistry in product **73**

Biohydroxylation of N-carboxybenzyl-cis-2,6-dimethylpiperidine 74

N-carboxybenzyl-cis-2,6-dimethyl-trans-4-hydroxypiperidine **75** was isolated as a colourless oil (10 mg, 5 %); δ_H (360 MHz) 1.25 (6H, d, 2 x CH_3, *J* 7), 1.42 - 1.60 & 1.86 - 1.90 (5H, m, C(3)H_2, C(4)OH & C(5)H_2), 4.20 (1H, tt, C(4)H, *J* 4 & 11), 4.49 - 4.57 (2H, m, C(2)H*eq* & C(6)H*eq*), 5.14 (2H, s, CH_2Ph), 7.23 - 7.38 (5H, m, aromatic); δ_C (63 MHz, DEPT) 21.7 (q, 2 x CH_3), 39.4 (t, C3 & C5), 47.2 (d, C2 & C6), 61.4 (d, C4), 66.9 (t, CH_2Ph), 127.7 (d, aromatic), 127.8 (d, aromatic), 128.4 (d, aromatic); *m/z* (EI) required 263.15214, found 263.15203, (FAB) 264 (MH^+).

Biohydroxylation of N-carboxybenzyl-2-ethylpiperidine 76

N-carboxybenzyl-trans-2-ethyl-4-hydroxypiperidine **77** was isolated as a colourless oil (130 mg, 45 %); δ_H (360 MHz) 0.85 (3H, t, C(8)H_3, *J* 7.5), 1.23 - 1.66 (5H, m, C(3)H, C(4)OH, C(5)H &C(7)H_2), 1.92 (2H, dd, C(3)H & C(5)H, *J* 2.5 & 12.5,), 2.85 (1H, ddd, C(6)H*ax*, *J* 2.5, 13.5 & 13.5), 3.91 (1H, tt, C(4)H, *J* 4.5 & 11.5), 4.16 (1H, br. d, C(6)H*eq*, *J* 12.5), 4.33 (1H, br. m, C(2)H*eq*), 5.10 (2H, d, CH_AH_BPh, *J* 12.5), 5.13 (2H, d, CH_AH_BPh, *J* 12.5), 7.25 - 7.37 (5H, m, aromatic); δ_C (90 MHz) 10.7 (q, C8), 23.8 (t, C7), 35.0 (C3 & C5), 37.6 (t, C6), 52.8 (d, C2), 65.0 (d, C4), 67.0 (t, CH_2Ph), 127.6 (d, aromatic), 127.8 (d, aromatic), 128.3 (d, aromatic), 136.8 (s), 155.4 (s); *m/z* (EI) required

263.15214, found 263.15253, (APCi) 264 (12, MH^+), 172 (7, M - $PhCH_2$), 156 (23, M - $PhCH_2O$), 91 (100, $PhCH_2$).

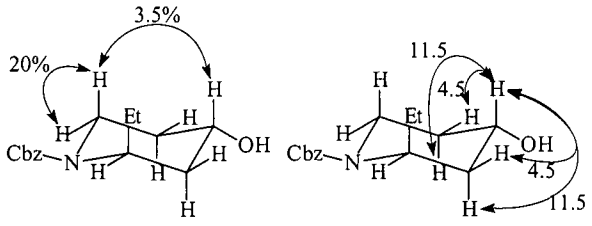


Figure 85: nOe enhancements and coupling constants used in assigning relative stereochemistry in product **77**

Biohydroxylation of N-carboxybenzyl-3,3-dimethylpiperidine 78

N-carboxybenzyl-3,3-dimethyl-4-hydroxypiperidine **79** was isolated as a colourless oil (134 mg, 48 %); δ_H (250 MHz @ 345K) 0.89 (3H, s) & 0.95 (3H, s) ($C(7)H_3$ & $C(8)H_3$), 1.51 - 1.64 (2H, m, C(5)H & C(4)OH), 1.71 - 1.82 (1H, m, C(5)H), 2.85 (1H, d, C(2)H*ax*, *J* 13.5), 3.12 - 3.23 (1H, m, C(6)H*ax*), 3.40 (1H, dd, C(4)H, *J* 4.0 & 8.5), 3.56 (1H, dd, C(2)H*eq*, *J* 1.5 & 13.5), 3.81 - 3.91 (1H, m, C(6)H*eq*), 5.13 (2H, s, CH_2Ph), 7.24 - 7.36 (5H, m, aromatic); δ_H (63 MHz) 18.4 (q, C7 or C8), 24.2 (q, C7 or C8), 29.5 (s, C3), 35.8 (t, C5), 41.5 (t, C2 or C6), 52.6 (t, C2 or C6), 66.9 (t, CH_2Ph), 74.7 (d, C4), 127.7 (d, aromatic), 127.8 (d, aromatic), 128.3 (d, aromatic), 136.7 (s), 155.4 (s); *m/z* (EI) required 263.15214, found 263.15254, 263 (10 %, M^+), 172 (5, M - $PhCH_2$), 128 (3, M -CO_2CH_2Ph), 91 (100, CH_2Ph), 77 (4, Ph).

5.3 *Rhodococcus* sp. NCIMB 9784

5.3.1 Maintenance and growth of microorganism

Rhodococcus sp. NCIMB 9784 was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, U.K.. The organism was maintained on agar plates at 4 °C and these were subcultured at regular intervals. A loop of bacteria was used to inoculate 100 mL of sterile basal salts medium[§] with 0.5 g L^{-1} ferrous sulfate heptahydrate and 4 g L^{-1} sodium pyruvate added through sterile filters, in a 250 mL Erlenmeyer flask. After three days growth on an orbital shaker at 250 r.p.m. at 30 °C, five such cultures were used to inoculate 8 L sterile basal salts media in a 10 L fermenter with 0.05 g L^{-1} ferrous sulfate heptahydrate and 1 g L^{-1} (+)-camphor added. The culture was grown for three days at 30 °C with aeration and stirring (200 rpm), with additional (+)-camphor (1 g L^{-1}) added after two days growth. The cells were harvested by centrifugation (11K r.p.m. for 30 minutes) and washed with phosphate buffer (50 mM, pH 7.4) to remove excess camphor.

5.3.2 General biotransformation procedure

Resting cell transformations were carried out by resuspending cell mass in one-tenth growth volume of phosphate buffer (50 mM, pH 7.0), the substrate added as a solution in ethanol to 0.5 mg mL^{-1} and the suspension incubated at 30 °C with shaking (250

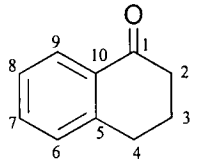
[§] Solution A: ammonium chloride 2.0 g L^{-1}; potassium dihydrogen phosphate 3.1 g L^{-1}, dipotassium hydrogen phosphate 8.2 g L^{-1}, yeast extract 0.1 g L^{-1}, tryptone 0.1 g L^{-1}; Solution B: magnesium sulfate 0.05 g L^{-1}, manganese sulfate 0.05 g L^{-1}, calcium chloride 0.01 g L^{-1}, ammonium molybdate 0.01 g L^{-1}. Solutions autoclaved separately and mixed when cool.

r.p.m.). The reaction was monitored by GC analysis until utilisation of substrate ceased. The cells were removed from the suspension by centrifugation (11K r.p.m. for 30 minutes) and the supernatant extracted with ethyl acetate. The combined organic extracts were dried over magnesium sulfate and the resultant oil purified by wet flash chromatography using petroleum ether/ethyl acetate mixtures as eluent.

5.3.3 Inhibition experiments

In these experiments, an aliquot of inhibitor solution was added to the resting cell suspension at the same time as the substrate. For such small scale experiments, substrate was added to 0.1 mg per mL and inhibitor added to 2.5 mM concentration in an attempt to saturate the system with inhibitor. The suspension was incubated at 30 °C for 14 hours and extracted with ethyl acetate for GC analysis.

5.3.4 Biotransformation of 1,2,3,4-tetrahydronaphthalene (tetralin) 91



α-Tetralone **96** was isolated as a pale yellow oil 23 (81 mg, 37 %); δ_H (250 MHz): 2.06 - 2.16 (2H, m, C(2)H_2 or C(3)H_2), 2.60 - 2.65 (2H, m, C(2)H_2 or C(3)H_2), 2.94 (2H, dd, C(4)H_2, *J* 6 & 6,), 7.20 - 7.31 (2H, m, C(7)H & C(8)H), 7.44 (1H, ddd, C(6)H, *J* 1.5, 7.5 & 7.5), 7.99 - 8.02 (1H, m, C(9)H), δ_C (63 MHz): 23.1 (t, C3). 29.5 (t, C2), 39.0 (t, C4), 126.4 (d, C7 or C8), 127.0 (d, C7 or C8), 128.6 (d, C6), 132.4 (s, C5), 133.2 (d, C9), 144.3 (s, C10), 198.3 (s, C1); *m/z* (EI) required 146.07317, found 146.07271; 146 (65 %, M^+), 118 (100, M - CO). Identity confirmed by comparison to commercially available material.

5.4 *Rhodococcus rhodochrous* NCIMB 9703

5.4.1 Substrate synthesis

General benzylation procedure (A)

Alcohol (1 mol eq.) and tetrabutylammonium iodide (trace) were added to a solution of sodium hydride (60 % suspension in mineral oil; 1.33 mol eq.) and freshly distilled benzyl bromide (1.67 mol eq.) in anhydrous THF under an atmosphere of argon at 0 C. On complete utilisation of the alcohol, the reaction mixture was diluted with diethyl ether and washed with saturated sodium hydrogen carbonate, saturated brine and water. The organic extracts were dried over anhydrous $MgSO_4$, filtered, concentrated under reduced pressure and purified by flash chromatography.

General preparation of 2-oxy-substituted oxygen heterocycles (B)

The dihydroheterocycle (1.5 mol eq.) was added to a stirred solution of alcohol (1 mol eq.) and 4-toluenesulfonic acid (4 mol %) in anhydrous dichloromethane under an atmosphere of argon. On complete consumption of the alcohol, the reaction mixture was diluted with diethylether and washed with saturated brine, saturated sodium hydrogen carbonate and water. The organic extracts were then dried over anhydrous $MgSO_4$, filtered, concentrated under reduced pressure and purified by flash chromatography.

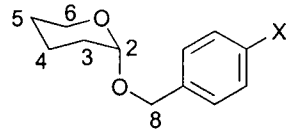
General esterification procedure using acid chloride (C)

Acid chloride (1.2 mol eq.) and pyridine (2 mol eq.) were added to a stirred solution of the alcohol (1 mol eq.) in anhydrous dichloromethane under an atmosphere of argon. Dimethylaminopyridine (trace) was also added if reaction was sluggish. On complete consumption of alcohol, the reaction mixture was diluted with dichloromethane and washed with citric acid (5 % aqueous solution), saturated sodium hydrogen carbonate solution, saturated brine and water. The organic extracts were dried over anhydrous $MgSO_4$, filtered, concentrated under reduced pressure and purified by flash chromatography.

General esterification procedure using DCC coupling method (D)

DCC (1 mol eq.) and acid (1.5 mol eq.) were added to a stirred solution of the alcohol (1 mol eq.), pyridine (1.5 mol eq.) and dimethylaminopyridine (catalytic) in anhydrous dichloromethane under an atmosphere of argon. On complete consumption of alcohol, the reaction mixture was diluted with ethyl acetate and filtered through cotton wool to remove dicyclohexylurea and the filtrate evaporated. The dilution, filtration and evaporation cycle was repeated twice to remove traces of DCU. The filtrate was then washed with citric acid (5 % aqueous solution), saturated sodium hydrogen carbonate solution, saturated brine and water. The organic extracts were dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure.

Typical numbering scheme of six membered oxygen heterocycles



2-benzyloxy-tetrahydropyran 112[117]

Acetal **112** was prepared using the general procedure **B** from benzylalcohol and dihydropyran.** ν_{max} 1120.3 (CO), 2867.8 & 2939.3 (CH stretch); δ_H (250 MHz) 1.50 – 1.91 (6H, m, C(3)H_2, C(4)H_2 & C(5)H_2), 3.55 (1H, m, C(6)H_A), 3.94 (1H, m, C(6)H_B), 4.51 (1H, d, C(8)H_A, *J* 12), 4.72 (1H, dd, C(2)H, *J* 3.5 & 3.5), 4.80 (1H, d, C(8)H_B, *J* 12), 7.32 (5H, m, aromatic); δ_C (63 MHz) 19.2 (t, C3, C4 or C5), 25.3 (t, C3, C4 or C5), 30.4 (t, C3, C4 or C5), 61.9 (t, C6 or C8), 68.6 (t, C6 or C8), 97.5 (d, C2), 127.3 (d, aromatic), 127.6 (d, aromatic), 128.2 (d, aromatic), 138.1 (s); *m/z* (EI) requires 192.11503, found 192.11517; 192 (3 %, M^+), 107 (16, $PhCH_2O$), 101 (39, M – $PhCH_2$), 91 (100, $PhCH_2$), 85 (43, M – $PhCH_2O$).

** Compound prepared by Dr. Gideon Grogan; purified and characterised by the author

2-(4-methylbenzyloxy)-tetrahydropyran 113

Acetal **113** was prepared following the general procedure from 4-methylbenzylalcohol and dihydropyran.** ν_{max} 1118.7 (CO), 2869.3 & 2490.5 (CH stretch); δ_H (250 MHz) 1.51 – 1.91 (6H, m, C(3)H_2, C(4)H_2 & C(5)H_2), 2.36 (3H, s, Me), 3.56 (1H, m, C(6)H_A), 3.95 (1H, m, C(6)H_B), 4.49 (1H, d, C(8)H_A, *J* 12), 4.72 (1H, dd, C(2)H, *J* 3.5 & 3.5), 4.76 (1H, d, C(8)H_B, *J* 12), 7.17 (2H, d, aromatic, *J* 8), 7.28 (2H, d, aromatic, *J* 8); δ_C (63 MHz) 19.2 (t), 21.0 (q, Me), 25.3 (t), 30.4 (t), 61.9 (t, C6 or C8), 68.5 (t, C6 or C8), 97.3 (d, C2), 127.8 (d, aromatic), 128.8 (d, aromatic), 135.1 (s), 136.9 (s); *m/z* (EI) requires 206.13068 found 206.11321; 206 (2 %, M^+), 105 (100, $CH_3C_6H_4CH_2$), 91 (16, $PhCH_2$), 85 (29, M – $CH_3C_6H_4CH_2O$).

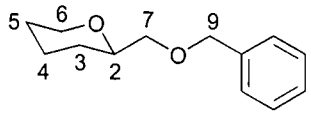
2-(4-chlorobenzyloxy)-tetrahydropyran 116

Acetal **116** prepared following the general method **B** from dihydropyran and 4-chlorobenzylalcohol.** ν_{max} 1089.5 (CO), 2880.9 & 2978.1 (CH); δ_H (250 MHz) 1.49 – 1.87 (6H, m, C(3)H_2, C(4)H_2 & C(5)H_2), 3.53 (1H, m, C(6)H_A), 3.88 (1H, m, C(6)H_B), 4.45 (1H, d, C(8)H_A, *J* 12), 4.68 (1H, dd, C(2)H, *J* 3 & 3), 4.73 (1H, d, C(8)H_B, *J* 12), 7.27 (4H, m, aromatic); δ_C (63 MHz) 19.1 (t, C3, C4 or C5), 25.3 (t, C3, C4 or C5), 30.3 (t, C3, C4 or C5), 62.0 (t, C6 or C8), 67.8 (t, C6 or C8), 97.6 (d, C2), 128.3 (d, aromatic), 128.9 (d, aromatic), 133.0 (s), 136.6 (s); *m/z* (EI) requires 226.07606 and 228.07311, found 226.07604 and 228.07357; 228 (2%, ^{37}Cl M^+), 226 (7, ^{35}Cl, M^+), 127 (47, $^{37}ClC_6H_4CH_2$), 125 (100, $^{35}ClC_6H_4CH_2$), 101 (27, M – $ClC_6H_4CH_2$). Although this compound has been described previously in the literature, its characterisation has not been reported.

2-(4-nitrobenzyloxy)-tetrahydropyran 119

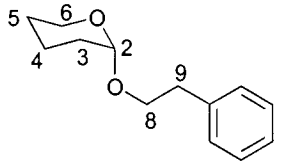
Acetal **119** was prepared following the general procedure **C** from 4-nitrobenzylalcohol and dihydropyran in 72 % yield (2.90 g). ν_{max} 1125.8 (CO), 1344.8 & 1520.8 (NO), 2866.1 & 2942.3 (CH stretch); δ_H (250 MHz) 1.50 – 1.90 (6H, m, C(3)H_2, C(4)H_2 & C(5)H_2), 3.52 (1H, m, C(6)H_A), 3.85 (1H, m, C(6)H_B), 4.57 (1H, d, C(8)H_A, *J* 13.5), 4.70 (1H, dd, C(2)H, *J* 3.5 & 3.5), 4.84 (1H, d, C(8)H_B, *J* 13.5), 7.50 (2H, d, aromatic, *J* 9), 8.16 (2H, d, aromatic, *J* 9); δ_C (63 MHz) 19.1 (t, C3, C4 or C5), 25.1 (t, C3, C4 or C5), 30.2 (t, C3, C4 or C5), 62.1 (t, C6 or C8), 67.4 (t, C6 or C8), 98.1 (d, C2), 123.3 (d, aromatic), 127.5 (d, aromatic), 145.9 (s), 147.0 (s); *m/z* (EI) requires 237.10011, found 237.10016; 237 (3 %, M^+), 136 (46, $O_2NC_6H_4CH_2$), 101 (34, M – $O_2NC_6H_4CH_2$), 85 (100, M – $O_2NC_6H_4CH_2O$).

2-benzyloxymethyl-tetrahydropyran 122[118]



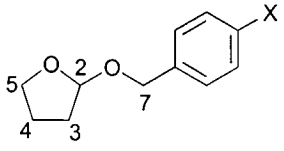
Ether **122** was prepared following the general benzylation procedure from (±)-tetrahydropyran-2-methanol and benzyl bromide.** ν_{max} 1090.9 (CO), 2849.8 & 2933.5 (CH stretch); δ_H (250 MHz) 1.45 – 1.62 & 1.83 (6H, m, C(3)H_2, C(4)H_2 & C(5)H_2), 3.45 (4H, m, C(6)H_2 & C(7)H_2), 4.03 (1H, m, C(2)H), 4.53 (1H, d, C(9)H_A, *J* 12), 4.60 (1H, d, C(9)H_B, *J* 12), 7.29 (5H, m, aromatic); δ_C (63 MHz) 23.0 (t, C3, C4 or C5), 25.8 (t, C3, C4 or C5), 28.1 (t, C3, C4 or C5), 68.3 (t, C6, C7 or C9), 73.2 (C6, C7 or C9), 73.6 (t, C6, C7 or C9), 76.7 (d, C2), 127.4 (d, aromatic), 127.6 (d, aromatic), 128.2 (d, aromatic), 138.1 (s); *m/z* (EI) required 206.13068, found 206.13064; 206 (3 %, M^+), 91 (23, $PhCH_2$), 85 (34, M – $PhCH_2OCH_2$), 77 (14, Ph).

2-phenethyloxy-tetrahydropyran 124



Acetal **124** was prepared following the general procedure **B** from 2-phenylethanol and dihydropyran in 65 % yield (1.65 g). ν_{max} 1070.9 (CO), 2867.0 & 2937.4 (CH stretch); δ_H (250 MHz) 1.46 – 1.85 (6H, m, C(3)H_2, C(4)H_2 & C(5)H_2), 2.92 (2H, dd, C(8)H_2, *J* 7 & 7), 3.45 (1H, m, C(6)H_A), 3.62 (1H, ddd, C(9)H_A, *J* 7, 7 & 9.5), 3.75 (1H, m, C(6)H_B), 3.94 (1H, ddd, C(9)H_B, *J* 7, 7 & 9.5), 4.60 (1H, dd, C(2)H, *J* 3 & 4), 7.16 – 7.32 (5H, m, aromatic); δ_C (63 MHz) 19.4 (t), 25.3 (t), 30.5 (t), 36.2 (t), 62.1 (t), 68.1 (t), 98.6 (d, C2), 126.0 (d, aromatic), 128.1 (d, aromatic), 128.9 (d, aromatic), 140.0 (s); *m/z* (EI) required 206.13068,, found 206.13097; 206 (0.2 %, M^+), 105 (73, $PhCH_2CH_2$), 101 (21, M – $PhCH_2CH_2$), 91 (16, $PhCH_2$), 85 (100, M – $PhCH_2CH_2O$). Although this compound has been described previously in the literature, its characterisation has not been reported.

Typical numbering scheme for five membered oxygen heterocycles



2-benzyloxy-tetrahydrofuran 125[119]

Acetal **125** was prepared following the general method from benzylalcohol and dihydrofuran in 86 % yield (2.14 g). ν_{max} 1090.1 (CO), 2880.2 & 2977.1 (CH stretch); δ_H (250 MHz) 1.80 – 2.09 (4H, m, C(3)H_2 & C(4)H_2), 3.93 (2H, m, C(5)H_2), 4.48 (1H, d, C(7)H_A, *J* 12), 4.71 (1H, d, C(7)H_B, *J* 12), 5.22 (1H, dd, C(2)H_2, *J* 2 & 3.5), 7.31 (5H, m, aromatic); δ_C (63 MHz) 23.3 (t, C3 or C4), 32.2 (t, C3 or C4), 66.9 (t, C5 or

C7), 68.6 (t, C5 or C7), 103.0 (d, C2), 127.4 (d, aromatic), 127.7 (d, aromatic), 128.2 (d, aromatic), 138.2 (s); *m/z* (EI) requires 178.09938, found 178.09973; 178 (11 %, M^+), 107 (21, $PhCH_2O$), 91 (100, $PhCH_2$), 87 (25, M – $PhCH_2$), 77 (25, Ph), 71 (80, M – $PhCH_2O$).

2-(4-methylbenzyloxy)-tetrahydrofuran 127

Acetal **127** was prepared following the general procedure **B** from 4-methylbenzylalcohol and dihydrofuran.** ν_{max} 1118.4 (CO), 2878.7 & 2976.7 (CH stretching); δ_H (250 MHz) 1.83 – 2.08 (4H, m, $C(3)H_2$ & $C(4)H_2$), 2.36 (3H, s, Me), 3.94 (2H, m, $C(5)H_2$), 4.47 (1H, d, $C(7)H_A$, *J* 11.5), 4.70 (1H, d, $C(7)H_B$, *J* 11.5), 5.25 (1H, dd, C(2)H, *J* 2 & 3.5), 7.17 (2H, d, aromatic, *J* 8), 7.26 (2H, d, aromatic, *J* 8); δ_C (63 MHz) 20.9 (q, Me), 23.3 (t, C3 or C4), 32.1 (t, C3 or C4), 66.7 (t, C5 or C7), 68.4 (t, C5 or C7), 102.7 (d, C2), 127.8 (d, aromatic), 128.8 (d, aromatic), 135.1 (s), 136.9 (s); *m/z* (EI) required 192.11503 found 192.11500; 192 (8 %, M^+), 105 (100, $CH_3C_6H_4CH_2$), 91 (23, $PhCH_2$).

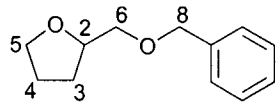
2-(4-chlorobenzyloxy)-tetrahydrofuran 129

Acetal **129** was prepared following the general method **C** from 4-chlorobenzylalcohol and dihydrofuran.** ν_{max} 806.3 (CCl), 1117.2 (CO), 2881.1 & 2977.5 (CH); δ_H (250 MHz) 1.80 – 2.05 (4H, m, $C(3)H_2$ & $C(4)H_2$), 3.90 (2H, m, $C(5)H_2$), 4.43 (1H, d, $C(7)H_A$, *J* 12), 4.66 (1H, d, $C(7)H_B$, *J* 12), 5.18 (1H, dd, C(2)H, *J* 2.5 & 3.5), 7.27 (4H, m, aromatic); δ_C (63 MHz) 23.3 (t, C3 or C4), 32.2 (t, C3 or C4), 66.9 (t, C5 or C7), 67.8 (t, C5 or C7), 103.1 (d, C2), 128.3 (d, aromatic), 129.0 (d, aromatic), 133.0 (s), 136.8 (s); *m/z* (EI) requires 212.06041 & 214.05746, found 212.06038 & 214.05747; 214 (7 %, ^{37}Cl M^+), 212 (17, ^{35}Cl M^+), 127 (48, $^{37}ClC_6H_4CH_2$), 125 (90, $^{35}ClC_6H_4CH_2$), 87 (27, M – $ClC_6H_4CH_2$), 71 (100, M – $ClC_6H_4CH_2O$).

2-(4-nitrobenzyloxy)-tetrahydrofuran 132

Acetal **132** was prepared following the general procedure **C** from 4-nitrobenzylalcohol and dihydrofuran in 71 % yield (2.69 g). ν_{max} 1110.3 (CO), 1344.9 & 1520.0 (NO), 2884.3 & 2978.0 (CH stretch); δ_H (200 MHz) 1.82 – 2.10 (4H, m, C(3)H_2 & C(4)H_2), 3.91 (2H, m, C(5)H_2), 4.56 (1H, d, C(7)H_A, *J* 13.5), 4.78 (1H, d, C(7)H_B, *J* 13.5), 5.22 (1H, m, C(2)H), 7.47 (2H, d, aromatic, *J* 9), 8.17 (2H, d, aromatic, *J* 9); δ_C (50 MHz) 23.3 (t, C3 or C4), 32.3 (t, C3 or C4), 67.1 (t, C5 or C7), 67.4 (t, C5 or C7), 103.5 (d, C2), 123.4 (d, aromatic), 127.6 (d, aromatic), 146.1 (s), 147.1 (s); *m/z* (EI) requires 223.08446 found 223.08449; 223 (3 %, M^+), 136 (78, $O_2NC_6H_4CH_2$).

2-(benzyloxymethyl)tetrahydrofuran 134[120]

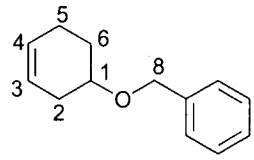


Ether **134** was prepared following the general benzylation procedure **A** using tetrahydrofurfuryl alcohol and benzyl bromide in a 46 % yield (0.87 g). ν_{max} 1083.9 (CO), 2858.7 & 2697.2 (CH stretch); δ_H (250 MHz) 1.62 (1H, m) & 1.90 (3H, m) (C(3)H_2 & C(4)H_2), 3.47 (2H, d, C(6)H_2, *J* 5), 3.76 (1H, m, C(5)H_A), 3.88 (1H, m, C(5)H_B), 4.07 (1H, m, C(2)H), 4.55 (1H, d, C(8)H_A, *J* 12), 4.59 (1H, d, C(8)H_B, *J* 12), 7.30 (5H, m, aromatic); δ_C (63 MHz) 25.5 (t, C3 or C4), 28.0 (t, C3 or C4), 68.1 (t), 72.6 (t), 73.2 (t), 77.7 (d, C2), 127.4 (d, aromatic), 127.5 (d, aromatic), 128.2 (d, aromatic), 138.2 (s); *m/z* (EI) required 192.11503 found 192.11516; 192 (M+), 107 ($PhCH_2O$), 101 (M – $PhCH_2$), 91 ($PhCH_2$), 71 (M – $PhCH_2OCH_2$).

Preparation of cyclohex-3,4-enol[121]

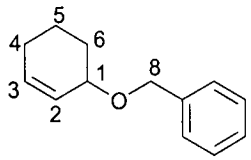
1,4-cyclohexanediol (15 g, 0.13 mol) and concentrated sulphuric acid (0.05 mL) were heated to 200 °C. The distillate collected was diluted with diethylether and dried over $MgSO_4$. The product was then purified by distillation at reduced pressure yielding alcohol (10 %). δ_H (200 MHz) 1.46 – 2.31 (6H, m, $C(2)H_2$, $C(5)H_2$ & $C(6)H_2$), 3.01 (1H, s, C(1)OH), 3.83 (1H, m, C(1)H), 5.52 (2H, m, C(3)H & C(4)H).

1-Benzyloxy-cyclohex-3,4-ene 144[121]



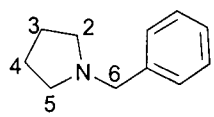
Ether **144** was prepared in a 75 % yield (1.56 g) following the general benzylation procedure **A** from cyclohex-3,4-en-1-ol. δ_H (250 MHz) 1.66 (1H, m), 1.94 – 2.26 (4H, m) & 2.40 (1H, m) ($C(2)H_2$, $C(5)H_2$ & $C(6)H_2$), 3.66 (1H, m, C(1)H), 4.58 (1H, d, $C(8)H_A$, *J* 12), 4.61 (1H, d, $C(8)H_B$, *J* 12), 5.62 (2H, m, C(3)H & C4(H)), 7.31 (5H, m, aromatic); δ_C (63 MHz) 23.9 (t, C2, C5 or C6), 27.7 (t, C2, C5 or C6), 31.6 (t, C2, C5 or C6), 69.8 (t, C8), 73.7 (d, C1), 124.2 (d), 126.7 (d), 127.3 (d), 127.4 (d), 128.2 (d), 138.9 (s); *m/z* (EI) requires 188.12012, found 188.12026; 188 (M^+), 107 ($PhCH_2O$), 91 ($PhCH_2$).

1-benzyloxy-cyclohex-2,3-ene 146[122]



Ether **146** was prepared in a 60 % yield (1.15 g) following the general benzylation procedure from cyclohex-2,3-en-1-ol. ν_{max} 1650.5 (C=C stretch), 2861.2 & 2933.3 (CH stretch), 3023.8 (C=CH stretch); δ_H (250 MHz) 1.57 – 2.07 (6H, m, C(4)H_2, C(5)H_2 & C(6)H_2), 3.97 (1H, m, C(1)H), 4.57 (1H, d, C(8)H_A, *J* 12), 4.63 (1H, d, C(8)H_B, *J* 12), 5.86 (2H, m, C(2)H & C(3)H), 7.32 (5H, m, aromatic); δ_C (63 MHz) 19.1 (t, C4, C5 or C6), 25.1 (t, C4, C5 or C6), 28.2 (t, C4, C5 or C6), 69.8 (t, C8), 72.0 (d, C1), 127.2 (d), 127.4 (d), 127.6 (d), 128.1 (d), 130.8 (d), 138.9 (s); *m/z* (EI) required 188.12012, found 188.12058; 188 (10 %, M^+), 107 (14, $PhCH_2O$), 91 (100, $PhCH_2$), 81 (32, M – $PhCH_2O$)

N-benzylpyrrolidine 150[111]



Amine **150** was prepared in 72 % yield (0.65 g) from pyrrolidine (1 mol eq.), benzyl bromide (1.1 mol eq.) and potassium hydroxide (1.1 mol eq.) in ethanol. ν_{max} 2961.0 (CH), 2781.4 (N*CH*$_2$); δ_H (250 MHz) 1.78 (4H, m, C(3)H_2 & C(4)H_2), 3.52 (4H, m, C(2)H_2 & C(5)H_2), 3.62 (2H, s, C(6)H_2), 7.29 (5H, m, aromatic); δ_C (63 MHz) 23.3 (t, C3 & C4), 54.0 (t, C2 & C5), 60.6 (t, C6), 126.7 (d, aromatic), 128.0 (d, aromatic), 128.8 (d, aromatic), 139.2 (s); *m/z* (EI) requires 161.12045, found 161.11994; 161 (65 %, M^+), 91 (100, $PhCH_2$), 70 (67, M – $PhCH_2$).

5.4.2 Maintenance and growth of microorganism

Rhodococcus rhodochrous NCIMB 9703 was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, U.K. The organism was maintained on agar plates at 4 °C and these were subcultured at regular intervals. A loop of bacteria was used to inoculate 50 mL of sterile basal salts medium[††] with 0.05 g L^{-1} ferrous sulfate heptahydrate and 4 g L^{-1} sodium pyruvate added *via* sterile filters, in a 250 mL Erlenmeyer flask. After three days agitation on an orbital shaker at 250 r.p.m. at 30° C, each culture was used to inoculate 500 mL of the same medium with 0.05 g L^{-1} ferrous sulfate heptahydrate and 5 mL octane per 500 mL culture added, in a 2 L Erlenmeyer flask. This culture was grown for five to seven days. The cells were harvested by centrifugation (10 000 rpm for 30 minutes) and washed with phosphate buffer (50 mM, pH 7.0).

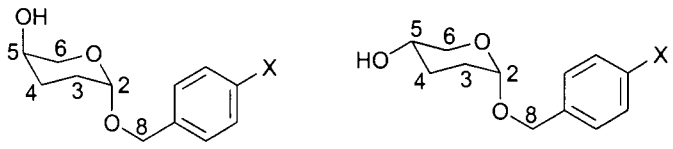
5.4.3 General biotransformation procedure

Resting cell transformations were carried out by re-suspending washed cells in one-tenth growth volume of phosphate buffer (50 mM, pH 7.0). A solution of 100 mg substrate in 1 mL ethanol was prepared and this was added to the resting cell culture (200 mL) of *Rhodococcus rhodochrous* NCIMB 9703. After 16 hours incubation as above, the cells were removed by centrifugation and the supernatant extracted into ethyl acetate. Purification of metabolites was routinely carried out using flash silica chromatography with petroleum ether/ethyl acetate or dichloromethane/ethyl acetate as eluent.

[††] Solution A: ammonium chloride 2.0 g L^{-1}; potassium dihydrogen phosphate 3.1 g L^{-1}, dipotassium hydrogen phosphate 8.2 g L^{-1}, yeast extract 0.1 g L^{-1}, tryptone 0.1 g L^{-1}; Solution B: magnesium sulfate 0.05 g L^{-1}, manganese sulfate 0.05 g L^{-1}, calcium chloride 0.01 g L^{-1}, ammonium molybdate 0.01 g L^{-1}. Solutions autoclaved separately and mixed when cool.

5.4.4 Biohydroxylation results

Product structures and numbering schemes



A **B**

Typical products identified from biohydroxylation with *Rhodococcus rhodochrous* NCIMB 9703: *trans*-2-aryl-5-hydroxytetrahydropyran **A** and *cis*-2-aryl-5-hydroxytetrahydropyran **B**.

Biohydroxylation of 2-(4-methylbenzyloxy)tetrahydropyran 113

trans-2-(4-methylbenzyloxy)-5-hydroxytetrahydropyran **114** was isolated as a colourless oil (10 mg, 9 %). $[\alpha_D]$ -21° (c 1.4); δ_H (360 MHz) 1.60 (2H, m, C(3)H_A & C(4)H_A), 2.04 (2H, m, C(3)H_B & C(4)H_B), 2.34 (3H, s, Me), 3.47 (1H, dddd, C(6)H*eq*, *J* 0.5, 2, 3.5 & 12), 3.80 (1H, br. s, C(5)H), 4.00 (1H, dd, C(6)H*ax*, *J* 2 & 12), 4.46 (1H, d, C(8)H_A, *J* 12), 4.70 (1H, d, C(8)H_B, *J* 12), 4.78 (1H, dd, C(2)H, *J* 0.5 & 2.5), 7.18 (4H, m, aromatic), δ_C (90 MHz) 21.6 (q, CH_3), 25.6 (t, C3 or C4), 26.2 (t, C3 or C4), 65.4 (d, C5), 65.6 (t, C6 or C8), 76.4 (t, C6 or C8), 97.0 (d, C2), 128.4 (d, aromatic), 129.5 (d, aromatic), 135.3 (s), 137.8 (s); *m/z* (EI) required 222.12559, found 222.12559; 222 (0.8 %, M^+), 121 (100, CH_3PhCH_2O), 91 (58, $PhCH_2$).

cis-2-(4-methylbenzyloxy)-5-hydroxytetrahydropyran **115** was isolated as a colourless oil (20 mg, 18 %). $[\alpha_D]$ +7° (c 1.0); δ_H (360 MHz, C_6D_6) 1.44 (3H, m, C(5)OH, C(3)H_A, C(4)H_B), 1.70 (2H, m, C(3)H_B, C(4)H_B), 2.11 (3H, s, CH_3), 3.39 (1H, m, C(5)H), 3.48 (1H, ddd, C(6)H*eq*, *J* 1.7, 4.3 & 10.7), 3.54 (1H, dd, C(6)H*ax*, *J* 8.8 & 10.7), 4.37 (1H, d, C(8)H_A, *J* 12), 4.59 (1H, dd, C(2)H, *J* 2.6 & 3), 4.71 (1H, d, C(8)H_B, *J* 12), 7.01 (2H,

d, aromatic, *J* 8), 7.25 (2H, d, aromatic, *J* 8); δ_C (93 MHz) 21.6 (q, CH_3), 28.6 (t, C3 or C4), 30.1 (t, C3 or C4), 66.0 (d, C5), 66.5 (t, C6 or C8), 69.3 (t, C6 or C8), 96.7 (d, C2), 128.5 (d, aromatic), 129.5 (d, aromatic), 135.2 (s, C12), 137.9 (s, C9); *m/z* (EI) required 222.12559, found 222.12634; 222 (8%, M^+), 121 (4, M - OH), 118 (12, M – CH_3PhCH), 105 (45, CH_3PhCH_2).

Biohydroxylation of 2-(4-chlorobenzyloxy)tetrahydropyran 116

trans-2-(4-chlorobenzyloxy)-5-hydroxytetrahydropyran **117** was isolated as a colourless oil (9 mg, 8 %). $[\alpha_D]$ –11 (c 1.6); δ_H (250 MHz) 1.60 (2H, m, C(3)H_A & C(4)H_A), 2.04 (2H, m, C(3)H_B & C(4)H_B), 3.48 (1H, m, C(6)H*eq*), 3.81 (1H, br. m, C(5)H), 3.97 (1H, dd, C(6)H*ax*, *J* 2 & 12), 4.47 (1H, d, C(8)H_A, *J* 12), 4.70 (1H, d, C(8)H_B, *J* 12), 4.77 (1H, dd, C(2)H, *J* 2.5 & 2.5), 7.25 - 7.33 (4H, m, aromatic); δ_C (63 MHz) 25.0 (t, C3 or C4), 25.6 (t, C3 or C4), 64.8 (d, C5), 65.1 (t, C6 or C8), 68.1 (t, C6 or C8), 96.6 (d, C2), 128.4 (d, aromatic), 129.0 (d, aromatic), 133.3 (s), 136.3 (s); *m/z* (EI) required 242.07097 and 244.06802, found 242.07065 and 244.06782; 244 (0.1 %, M^+ ^{37}Cl), 242 (0.5 %, M^+ ^{35}Cl), 127 (52, $^{37}ClC_6H_4CH_2$), 125 (100, $^{35}ClC_6H_4CH_2$), 91 (15, $PhCH_2$).

cis-2-(4-chlorobenzyloxy)-5-hydroxytetrahydropyran **118** was isolated as a colourless oil (13 mg, 12 %). δ_H (360 MHz) 1.71 - 1.95 (5H, m, C(3)H_2, C(4)H_2 & C(5)OH), 3.67 (2H, br. m, C(6)H_2), 3.79 (1H, br. m, C(5)H), 4.50 (1H, d, C(8)H_A, *J* 12.2), 4.72 (1H, m, C(2)H), 4.77 (1H, d, C(8)H_B, *J* 12.2), 7.28 - 7.38 (4H, m, aromatic); δ_C (63 MHz) 27.9 (t, C3 & C4), 65.3 (d, C5), 66.0 (t, C6 or C8), 68.0 (t, C6 or C8), 96.3 (d, C2), 128.4 (d, aromatic), 129.0 (d, aromatic), 133.3 (s), 136.2 (s); *m/z* 242 (M^+).

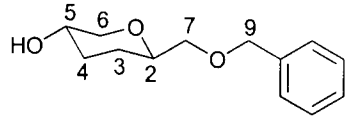
Biohydroxylation of 2-(4-nitrobenzyloxy)tetrahydropyran 119

trans-2-(4-nitrobenzyloxy)-5-hydroxytetrahydropyran **120** was isolated a colourless oil (22 mg, 21 %). $[\alpha_D]$ +36 (c 1.15); δ_H (360 MHz) 1.70 (2H, m, C(3)H_A & C(4)H_A), 1.70

(1H, br. s, C(5)OH), 2.13 (2H, m, C(3)H_B & C(4)H_B), 3.53 (1H, m, C(6)H*eq*), 3.99 (1H, dd, C(6)H*ax*, *J* 2.2 & 11.8), 4.65 (1H, d, C8-H_A, *J* 13.5), 4.85 (1H, m, C(2)H), 4.89 (1H, d, C(8)H_B, *J* 13.5), 7.55 (2H, d, aromatic, *J* 8.8), 8.24 (2H, d, aromatic, *J* 8.8); δ_C (90 MHz) 25.5 (t, C3 or C4), 26.2 (t, C3 or C4), 65.2 (d, C5), 65.7 (t, C6 or C8), 68.3 (t, C6 or C8), 97.7 (d, C2), 124.1 (d, aromatic), 128.2 (d, aromatic), 146.1 (s), 147.8 (s); *m/z* (EI) required 253.09502, found 253.09509; 253 (1%, M^+), 236 (4, M – OH), 153 (92, $O_2NC_6H_4CH_2OH$), 152 (44, $O_2NC_6H_4CH_2O$).

cis-2-(4-nitrobenzyloxy)-5-hydroxytetrahydropyran **121** was isolated as a pale yellow oil (19 mg, 18 %). [α_D] -44 (c 0.7); δ_H (360 MHz) 1.88 (5H, m, C(3)H_2, C(4)H_2 & C(5)OH), 3.63 (1H, dd, C(6)H*ax*, *J* 8 & 11), 3.70 (1H, ddd, C(6)H*eq*, *J* 1.2, 4 & 11), 3.80 (1H, m, C(5)H), 4.63 (1H, d, C(8)H_A, *J* 13.4), 4.76 (1H, dd, C(2)H, *J* 3.1 & 3.3), 4.90 (1H, d, C(8)H_B, *J* 13.4), 7.55 (2H, m, aromatic), 8.24 (2H, m, aromatic); δ_C (63 MHz) 27.8 (t, C3 or C4), 29.5 (t, C3 or C4), 65.2 (d, C5), 66.0 (t, C6 or C8), 67.6 (t, C6 or C8), 96.8 (d, C2), 123.5 (d, aromatic), 127.7 (d, aromatic), 145.5 (s), 147.7 (s); *m/z* (EI) required 253.09502, found 253.09501; 253 (0.1 %, M^+), 153 (21, $O_2NC_6H_4CH_2OH$).

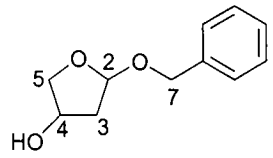
***Biohydroxylation of 2-(benzyloxymethyl)tetrahydropyran* 122**



trans-2-(benzylmethyloxy)-5-hydroxytetrahydropyran **123** was isolated as a colourless oil (38 mg, 35 %). [α_D] +2.9 (c 1.0); δ_H (360 MHz) 1.38 – 1.72 (4H, m) & 2.12 (1H, m) (C(5)OH, C(3)H_2 & C(4)H_2), 3.13 (1H, dd, C(6)H*ax*, *J* 10.4 & 10.6), 3.39 – 3.50 (3H, overlapping m, C(7)H_2 & C(2)H), 3.70 (1H, m, C(5)H), 4.03 (1H, ddd, C(6)H*eq*, *J* 2.2, 4.8 & 10.6), 4.53 (1H, d, C(8)H_A, *J* 12.2), 4.58 (1H, d, C(8)H_B, *J* 12.2), 7.29 (5H, m, aromatic); δ_C (90 MHz) 27.0 (t, C3 or C4), 32.4 (t, C3 or C4), 66.2 (d, C2), 72.6 (t, C6,

C7 or C8), 72.7 (t, C6, C7 or C8), 73.3 (t, C6, C7 or C8), 76.4 (d, C5), 127.5 (d, aromatic), 127.7 (d, aromatic), 128.8 (d, aromatic), 138.0 (s); *m/z* (EI) required 222.12559, found 222.12568; 222 (20 %, M^+), 205 (2, M – OH), 107 (30, $PhCH_2O$), 91 (62, $PhCH_2$).

***Biohydroxylation of 2-(benzyloxy)tetrahydrofuran* 125**



2-(benzyloxy)-4-hydroxytetrahydrofuran **126** was isolated as colourless oil (16 mg, 15 %). $[\alpha_D]$ +45 (c 0.6); δ_H (360 MHz) 1.95 (1H, br.s, C(4)OH), 2.14 (1H, dddd, $C(3)H_A$, *J* 1, 2.2, 5.5 & 14.4). 2.29 (1H, ddd, $C(3)H_B$, *J* 2.6, 6.4 & 14.4), 3.87 (1H, d, C(5)H, *J* 9.8), 4.04 (1H, dd, C(5)H, *J* 4.2 & 9.8), 4.53 (1H, d, $C(7)H_A$, *J* 11.8), 4.59 (1H, br. m, C(4)H), 4.77 (1H, d, $C(7)H_B$, *J* 11.8), 5.43 (1H, dd, C(2)H, *J* 2.5 & 5.5), 7.34 (5H, m, aromatic); δ_C (90 MHz) 43.3 (t, C3), 69.9 (t, C5 or C7), 72.1 (d, C4), 74.3 (t, C5 or C7), 103.6 (d, C2), 128.1 (d, aromatic), 128.3 (d, aromatic), 128.8 (d, aromatic), 138.3 (s); *m/z* (EI) required 194.09429, found 194.09434; 194 (0.7 %, M^+), 177 (0.9, M – OH), 107 (16, $PhCH_2O$), 103 (20, M – $PhCH_2$), 91 (100, $PhCH_2$), 77 (20, Ph).

***Biohydroxylation of 2-(4-methylbenzyloxy)tetrahydrofuran* 127**

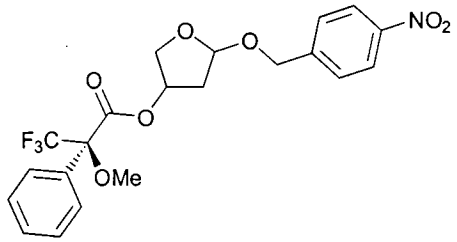
2-(4-methylbenzyloxy)-4-hydroxytetrahydrofuran **128** was isolated as a colourless oil (19 mg, 18 %). $[\alpha_D]$ +29° (c 0.25); δ_H (360 MHz) 2.13 (1H, dddd, $C(3)H_A$, *J* 1.5, 2.5, 5.5 & 14.5), 2.27 (1H, dddd, $C(3)H_B$, *J* 0.5, 2.5, 6 & 14.5), 2.37 (3H, s, CH_3), 3.87 (1H, dddd, $C(5)H_A$, *J* 0.5, 1.5, 1.5 & 10), 4.04 (1H, ddd, $C(5)H_B$, *J* 0.5, 4 & 10), 4.49 (1H, d, $C(7)H_A$, *J* 11.5), 4.60 (1H, m, C(4)H), 4.72 (1H, d, $C(7)H_B$, *J* 11.5), 5.42 (1H, dddd, C(2)H, *J* 0.5, 0.5, 2.5 & 5.5), 7.18 (2H, m, aromatic), 7.25 (2H, m, aromatic); δ_C (93 MHz) 21.0 (q, CH_3), 42.8 (t, C3), 69.2 (t, C5 or C7), 71.7 (d, C4), 73.7 (t, C5 or C7),

102.8 (d, C2-H), 127.9 (d, aromatic), 128.9 (d, aromatic), 134.7 (s), 137.3 (s); *m/z* (EI) required 208.10994, found 208.11008; 208 (8%, M^+), 191 (4, M – OH), 92 (35, $PhCH_3$), 91 (83, $PhCH_2$).

***Biohydroxylation of 2-(4-nitrobenzyloxy)tetrahydrofuran* 132**

2-(4-nitrobenzyloxy)-4-hydroxytetrahydrofuran **133** was isolated as a pale yellow oil (28 mg, 26 %). $[\alpha_D]$ +80 (c 1.5); δ_H (250 MHz, $CDCl_3$) 1.97 (1H, br m, C(4)OH), 2.15 (1H, dddd, C(3)H_A, *J* 1.2, 2.6, 5.4 & 14.4), 2.27 (1H, ddd, C(3)H_B, *J* 2.6, 6.0 & 14.4), 3.83 (1H, ddd, C(5)H_A, *J* 1.5, 1.5 & 10), 3.96 (1H, ddd, C(5)H_B, *J* 0.5, 4 & 10), 4.57 (1H, d, C(7)H_A, *J* 13), 4.58 (1H, m, C(4)H), 4.80 (1H, d, C(7)H_B, *J* 13), 5.38 (1H, dd, C(2)H, *J* 2.5 & 5.5), 7.46 (2H, d, *J* 8.8, aromatic), 8.17 (2H, d, *J* 8.8, aromatic); δ_C (63 MHz) 42.7 (t, C3), 68.1 (t, C5 or C7), 71.4 (d, C4), 73.9 (t, C5 or C7), 103.6 (d, C2-H), 123.5 (d, aromatic), 127.7 (d, aromatic), 145.6 (s), 147.2 (s); *m/z* (EI) required 239.07937, found 239.07941; 239 (4%, M^+), 136 (22, $O_2NC_6H_4CH_2$), 107 (31, PhCH2O).

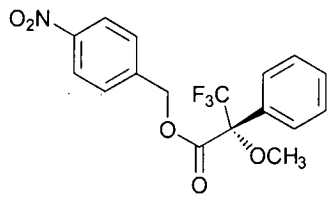
***2-(4-nitrobenzyloxy)-tetrahydrofur-4-yl-(α-trifluoromethyl-α-methoxyphenylacetate) (MTPA ester)* 135**



Ester **135** prepared following esterification method **D** from alcohol **133** and α-trifluoromethyl-α-methoxyphenyl acetic acid. $[\alpha_D]$ +37 (c 1.2); δ_H (250 MHz) 2.23 (1H, dddd, C(3)H_A, J 1, 2.5, 5.5, 15), 2.42 (1H, ddd, C(3)H_B, J 2.5, 7.0 & 15), 3.54 (3H, ddd, OMe, J_{HF} 1), 4.07 (2H, overlapping m, C(5)H_2), 4.58 (1H, d, C(7)H_A, J 13), 4.80 (1H, d, C(7)H_B, J 13), 5.35 (1H, dd, C(2)H, J 2.5 & 5.5), 5.58 (1H, m, C(4)H), 7.25 – 7.52 (7H, m, aromatic), 8.18 (2H, d, aromatic, J 8.8); δ_C (90 MHz) 39.8 (t, C3), 55.8 (q, OMe),

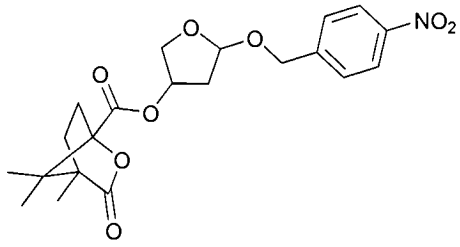
68.7 (t, C5 or C7), 71.3 (t, C5 or C7), 76.9 (d, C4), 103.7 (d, C2), 120.0 (s), 124.1 (d, aromatic), 125.2 (s), 127.6 (d, aromatic), 128.3 (d, aromatic), 129.0 (d, aromatic), 130.2 (d, aromatic), 132.3 (s), 145.7 (s), 147.8 (s), 166.8 (s); δ_F (235 MHz) –76.1 & -76.2, m/z (EI) required 455.11919 found 455.11922; 455 (0.2 %, M+), 319 (1, M – $O_2NC_6H_4CH_2$), 222 (4, M – $PhC(CF_3)(OMe)CO_2$), 189 (100, $PhC(CF_3)OMe$), 136 (53, $O_2NC_6H_4CH_2$).

***(4-nitrobenzyl)-α-trifluoromethyl-α-methoxy-phenylacetate* 136**



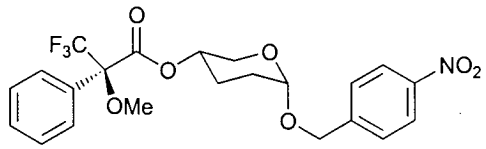
Ester **136** was prepared following the DCC coupling method D from α-trifluoromethyl-α-methoxyphenylacetic acid and 4-nitrobenzylalcohol in 90 % yield (50 mg). δ_H (250 MHz) 3.52 (3H, ddd, OMe, J_{HF} 1.5), 5.38 (1H, d, $PhCH_A$, *J* 13), 5.45 (1H, d, $PhCH_B$, *J* 13), 7.25 – 7.48 (7H, m, aromatic), 8.20 (2H, d, aromatic, *J* 9); δ_C (63 MHz) 55.4 (q, OMe), 66.1 (t, $PhCH_2O$), 120.8 (s), 123.8 (d, aromatic), 125.4 (s), 127.1 (d, aromatic), 128.4 (d, aromatic), 129.7 (d, aromatic), 131.6 (s), 141.5 (s), 166.1 (s); δ_F (235 MHz) -75.9, *m/z* (FAB) 370 (3 %, MH^+), 189 (100, $PhC(CF_3)(OMe)$), 136 (50, $O_2NC_6H_4CH_2$), 91 (48, $PhCH_2$), 77 (65, Ph).

2-(4-nitrobenzyloxy)-tetrahydrofur-4-yl camphanoate **138**



Ester **138** prepared following esterification method C from alcohol **133** and camphanic acid chloride (76 %). Found C, 60.0; H, 6.2; N, 3.2. $C_{12}H_{25}NO_8$ requires C, 60.1; H, 6.0; N, 3.3; δ_H (360 MHz) 0.99 (3H, s, Me), 1.08 (3H, s, Me), 1.15 (3H, s, Me), 1.72 (1H, m), 1.95 (1H, m), 2.06 (1H, m), 2.32 – 2.51 (3H, m)(C(3)H_2, 2 x camphane CH_2), 3.98 (1H, d, C(5)H_A, *J* 10.8), 4.14 (1H, dd, C(5)H_B, *J* 4.5 & 10.8), 4.64 (1H, d, C(7)H_A, *J* 13.2), 4.85 (1H, d, C(7)H_B, *J* 13.2), 5.44 (1H, dd, C(2)H, *J* 2.3 & 5.5), 5.53 (1H, m, C(4)H), 7.51 (2H, m, aromatic), 8.25 (2H, m, aromatic); *m/z* (EI) required 419.15802, found 419.15727; 419 (0.8%, M^+), 238 (4, ester cleaved), 137 (59, $O_2NC_6H_4CH_2O$).

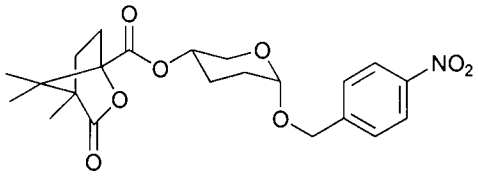
cis-2-(4-nitrobenzyloxy)-tetrahydropyr-5-yl-(α-trifluoromethyl–α-methoxy-phenylacetate) **139**



Ester **139** was prepared following the DCC coupling method D from alcohol **121** and α-trifluoromethyl-α-methoxyphenylacetic acid. [α_D] +8° (c 0.65) δ_H (250 MHz) 1.93 (4H, m, C(3)H_2 & C(4)H_2), 3.54 (3H, ddd, OMe, J_{HF} 1.2), 3.77 (2H, m, C(6)H_2), 4.58 (d, C(8)H_A', *J* 13.5), 4.60 (d, C(8)H_A, *J* 13.5), 4.75 (1H, m, C(2)H or C(5)H), 4.85 (1H, m, C(8)H_B), 5.07 (1H, m, C(2)H or C(5)H), 7.46 (7H, m, aromatic), 8.19 (2H, d, aromatic, *J* 8.8); δ_C (63 MHz) 24.3 (t, C3 or C4), 27.7 (t, C3 or C4), 27.9 (t, C3' or C4'), 55.3 (q,

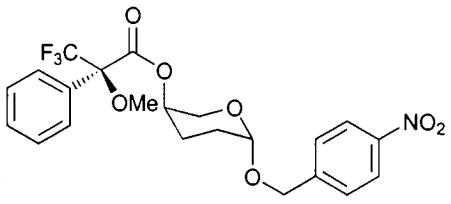
OMe), 61.5 (t, C6' or C8'), 62.3 (t, C6 or C8), 67.6 (t, C6' or C8'), 67.7 (t, C6 or C8), 69.6 (d, C2 or C5), 96.3 (s), 96.9 (d, C2 or C5), 120.8 (s), 123.5 (d, aromatic), 125.6 (s), 127.1 (d, aromatic), 127.6 (d, aromatic), 128.4 (d, aromatic), 129.6 (d, aromatic), 131.9 (s), 145.2 (s), 147.2 (s), 165.7 (s); δ_F (235 MHz) –72.1; *m/z* (EI) requires 470.14266, found 470.14267; 470 (0.6 %, M^+), 217 (56, PhC(OMe)(CF_3)C(O)), 189 (26, PhC(OMe)(CF_3)), 91 (86, $PhCH_2$).

***cis-2-(4-nitrobenzyloxo)-tetrahydropyr-5-yl camphanoate* 140**



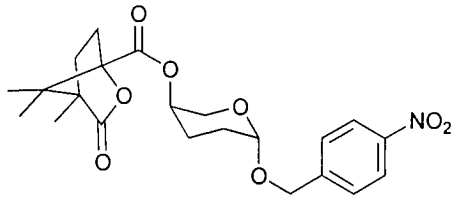
Ester **140** was prepared following the acid chloride method C from alcohol **121** and camphanic acid chloride.(61 %) δ_H (360 MHz) 0.95 (3H, s, CH_3), 1.04 (3H, s, CH_3), 1.11 (3H, s, CH_3), 1.68 (2H, m), 1.79 – 2.06 (5H, m) & 2.41 (1H, m) (C(3)H_2, C(4)H_2 & 2 x camphane CH_2), 3.74 (2H, m, C(6)H_2), 4.60 (1H, d, C(8)H_A, *J* 13.4), 4.76 (1H, m, C(2)H or C(5)H), 4.85 (1H, d, C(8)H_B, *J* 13.4), 4.97 (1H, m, C(2)H or C(5)H), 7.51 (2H, d, aromatic, *J* 8.8), 8.21 (2H, d, aromatic, *J* 8.8); δ_C (90 MHz) 10.1 (q, CH_3), 17.2 (q, CH_3), 17.3 (q, CH_3), 25.0 (t), 28.5 (t), 29.3 (t), 31.0 (t), 54.6 (s), 55.2 (s), 62.7 (t, C6 or C8), 68.3 (t, C6 or C8), 69.2 (d, C2 or C5), 91.4 (s), 97.2 (d, C2 or C5), 124.1 (d, aromatic), 128.2 (d, aromatic), 145.8 (s), 147.8 (s), 167.2 (s), 178.5 (s).

trans-2-(4-nitrobenzyloxy)-tetrahydropyr-5-yl-(α-methoxy-α-(trifluoromethyl)-phenylacetate) **141**



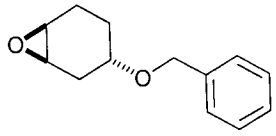
Ester **141** was prepared following the DCC coupling method D from alcohol **120** and α-trifluoromethyl-α-methoxyphenylacetic acid. [α_D] +38°(c 1.55) δ_H (250 MHz) 1.58 – 2.06 (3H, m) & 2.12 – 2.36 (1H, m) (C(3)H_2 & C(4)H_2), 3.53 (ddd, OMe, J_{HF} 1), 3.59 (ddd, OMe', J_{HF} 1), 3.70 (ddd, C(6)H_{eq}, *J* 2.0, 2.0 & 13), 3.78 (ddd, C(6)H_{eq}', *J* 2.0, 2.0 & 13), 3.95 (1H, dd, C(6)H_{ax}, *J* 2 & 13), 3.97 (1H, dd, C(6)H_{ax}', *J* 2 & 13), 4.60 (1H, d, C(8)H_A, *J* 13.5), 4.61 (1H, d, C(8)H_A', *J* 13.5), 4.83 (2H, m, C(8)H_B & C(2)H or C(5)H), 5.28 (1H, br. m, C(2)H or C(5)H), 7.37 – 7.88 (7H, m, aromatic); δ_C (63 MHz) 21.9 (t, C3 or C4), 22.2 (t, C3' or C4'), 24.6 (t, C3 or C4), 24.8 (t, C3' or C4'), 55.1 (q, OMe'), 55.3 (q, OMe), 61.0 (t, C6 or C8), 61.2 (t, C6' or C8'), 67.6 (t, C6 or C8), 69.9 (d, C2 or C5), 96.2 (d, C2 or C5), 96.3 (d, C2' or C5'), 121.0 (s), 123.6 (d), 125.6 (s), 127.1 (d), 127.4 (d), 127.7 (d), 128.3 (d), 129.5 (d), 131.8(s), 132.2 (s'), 145.3 (s), 147.3 (s), 165.9(s); 166.0 (s'); δ_F (235 MHz) –72.1 & -72.3; *m/z* (EI) requires 470.14266, found 470.14269; 470 (2 %, M^+), 217 (53, PhC(OMe)(CF_3)C(O)), 189 (37, PhC(OMe)(CF_3)), 91 (62, $PhCH_2$).

***trans-2-(4-nitrobenzyloxy)-tetrahydropyr-5-yl camphanate* 142**



Ester **142** was prepared following the acid chloride method C from alcohol **120** and camphanic acid chloride (58 %). δ_H (360 MHz) 0.97 (3H, s, CH_3'), 0.99 (3H, s, CH_3), 1.06 (3H, s, CH_3'), 1.09 (3H, s, CH_3), 1.11 (3H, s, CH_3), 1.62 – 2.25 (7H, m) & 2.44 (1H, m) (C(3)H_2, C(4)H_2 & 2 x camphane CH_2), 3.68 (1H, ddd, C(6)H_A, *J* 2.2, 2.2 & 12.8), 3.94 (1H, ddd, C(6)H_B, *J* 1.8, 1.8 & 12.8), 4.61 (1H, d, C(8)H_A, *J* 13.4), 4.82 (1H, d, C(8)H_B, *J* 13.4), 4.83 (1H, d, C(8)H_B', *J* 13.4), 4.88 (1H, m, C(2)H or C(5)H), 4.98 (1H, br. m, C(2)H or C(5)H), 7.50 (2H, d, aromatic, *J* 8.6), 8.20 (2H, d, aromatic, *J* 8.6); δ_C (90 MHz) 10.14 (q, CH_3), 10.18 (q, CH_3'), 17.1 (q, CH_3), 17.2 (q, CH_3), 22.7 (t), 25.5 (t), 29.3 (t), 29.4 (t'), 30.9 (t), 31.1 (t'), 54.6 (s'), 54.7 (s), 55.2 (s'), 55.3 (s), 62.0 (t, C6 or C8), 68.2 (t, C6 or C8), 69.3 (d, C2' or C5'), 69.4 (d, C2 or C5), 91.5 (s'), 91.6 (s), 96.9 (d, C2 or C5), 124.1 (d, aromatic), 128.2 (d, aromatic), 145.9 (s), 147.8 (s), 167.4 (s'), 167.5 (s), 178.5 (s'), 178.7 (s).

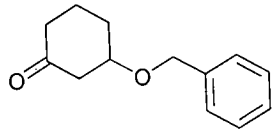
***Biohydroxylation of 4-benzyloxycyclohex-1,2-ene* 144**



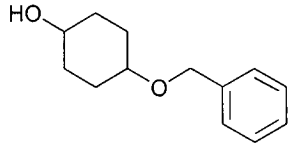
trans-4-benzyloxy-1,2-epoxycyclohexane **145**[121] was isolated as a colourless oil (40 %, 43 mg). [α_D] 3 (c 1.5); δ_H (250 MHz) 1.49 - 1.68 (2H, m) & 1.88 - 2.25 (4H, m) (C(2)H_2, C(5)H_2 & C(6)H_2 protons), 3.17 (2H, m, C(1)H & C(2)H), 3.53 (1H, m, C(4)H), 4.43 (1H, d, C(8)H_A, *J* 12), 4.52 (1H, d, C(8)H_B, *J* 12), 7.30 (5H, m, aromatic); δ_C (63 MHz) 20.5 (t, C5 or C6), 23.4 (t, C5 or C6), 30.4 (t, C3), 51.5 (d, C1 or C2), 52.0

(d, C1 or C2), 69.9 (t, C8), 70.5 (d, C4), 127.3 (d, aromatic), 128.3 (d, aromatic), 138.6 (s); *m/z* (EI) 204 (M^+).

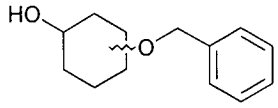
***Biohydroxylation of 4-benzyloxycyclohex-1,2-ene* 146**



3-(benzyloxy)-cyclohexanone **147**[123] was isolated as a colourless oil (36 mg, 33 %). [α_D] 0 (c 1); δ_H (250 MHz) 1.89 - 2.01 (2H, m, C(5)H_2), 2.09 - 2.31 (4H, m, C(4)H_2 & C(6)H_2), 2.62 (2H, m, C(2)H_2), 3.82 (1H, dddd, C(3)H, *J* 3, 3, 5.5 & 5.5), 4.60 (2H, s, C(8)H_2), 7.31 (5H, m, aromatic); δ_C (63 MHz) 30.4 (t, C5 & C6), 37.1 (t, C2 & C4), 70.1 (t, C8), 72.1 (d, C3), 127.3 (d, aromatic), 127.5 (d, aromatic), 128.3 (d, aromatic), 138.3 (s), 211.1 (s); *m/z* (EI) required 204.11503, found 204.11532; 204 (9%, M^+), 91 (100, $PhCH_2$).

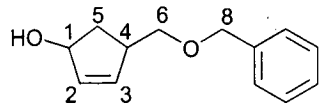


4-(benzyloxy)-cyclohexan-1-ol **148**[123] was isolated as a colourless oil (7 mg, 6 %). [α_D] 0 (c 0.5); δ_H (600 MHz) 1.57 (3H, m, C(1)OH & CH_2), 1.65 (2H, m, CH_2), 1.72 (2H, m, CH_2), 1.89 (2H, m, CH_2), 3.48 (1H, dddd, C(1)H or C(4)H, *J* 3.1, 3.1, 6.2 & 6.2), 3.73 (1H, dddd, C(1)H or C(4)H, *J* 4.0, 4.0, 8.0, 8.0), 4.52 (2H, s, C(8)H_2), 7.25 & 7.23 (5H, m, aromatic); δ_C (63 MHz) 27.4 (t, C2 & C6), 30.4 (t, C3 & C5), 68.4 (d, C1 or C4), 69.5 (t, C8), 73.3 (d, C1 or C4), 127.2 (d, aromatic), 127.3 (d, aromatic), 128.2 (d, aromatic), 139.0 (s); *m/z* (EI) required 206.13068, found 206.13070; 206 (M^+, 5 %), 205 (6, M-H), 91 (100, $PhCH_2$).



(Benzyloxy)-cyclohexan-1-ol **149** was isolated as a colourless oil (15 mg, 13 %). $[\alpha_D]$ 0 (c 0.5); δ_H (600 MHz) 1.23 - 1.61 (5H, m, C(1)OH, 2 x CH_2), 1.97 (2H, m, CH_2), 2.05 (2H, m, CH_2), 3.38 (1H, dddd, C(1)H or C*H*OCH_2), *J* 4.0, 4.0, 9.7 & 9.7), 3.68 (1H, m, C(1)H or C*H*OCH_2), 4.53 (2H, s, C(8)H_2), 7.26 & 7.33 (5H, m, aromatic); δ_C (63 MHz) 29.2 (t, C2 & C6), 32.6 (t, C3 & C5), 69.5 (d, C4), 70.0 (t, C8), 76.0 (d, C1), 127.3 (d, aromatic), 127.4 (d, aromatic), 128.2 (d, aromatic), 138.8 (s); *m/z* (EI) required 206.13068, found 206.13072; 206 (1 %, M^+), 205 (4, M-H), 91 (100, $PhCH_2$).

***Biohydroxylation of 3-benzyloxymethyl-cyclopent-1,2-ene* 152**



1-hydroxy-4-benzyloxymethyl-cyclopent-2,3-ene **153** was isolated as a colourless oil (19 %, 21 mg). $[\alpha_D]$ -25° (c 1.0); δ_H (360 MHz) 1.91 (1H, ddd, C(5)H_A, *J* 3.2, 7.8 & 14.2), 2.00 (1H, ddd, C(5)H_B, *J* 5, 7.2 & 14.2), 3.23 (1H, m, C(4)H), 3.39 (2H, m, C(6)H_2), 4.55 (2H, s, C(8)H_2), 4.92 (1H, m, C(1)H), 5.93 (1H, m, C(2)H or C(3)H), 6.04 (1H, ddd, C(2)H or C(3)H, *J* 0.8, 2 & 5.6), 7.39 (5H, m, aromatic); δ_C (90 MHz) 37.9 (t, C5), 45.3 (d, C4), 73.5 (t, C6 or C8), 74.4 (t, C6 or C8), 77.5 (d, C1), 128.0 (2 x d), 128.8 (d), 134.8 (d), 137.4 (d), 138.8 (s); *m/z* (EI) 204 (M^+), 113 (M – $PhCH_2$), 107 ($PhCH_2O$), 91 ($PhCH_2$), 83 (M – $PhCH_2OCH_2$), 77 (Ph).

6 References

1 S. M. Roberts and N. M. Williamson, *Curr. Org. Chem.*, 1997, **1**, 1.

2 F. H. Arnold, *Acc. Chem. Res.*, 1998, **31**, 125.

3 M. T. Reetz, A. Zonta, K. Schimossek, K. Liebeton, and K. E. Jaeger, *Angew. Chem., Int. Ed. Engl.*, 1997, **36**, 2830.

4 D. H. R. Barton, F. Hally, N. Ozbalik, M. Schmitt, E. Young, and G. Balaroine, *J. Am. Chem. Soc.*, 1989, **111**, 7144.

5 T. Yang and R. Breslow, *Angew. Chem., Int. Ed. Engl.*, 2000, **39**, 2692.

6 D. H. Peterson and H. C. Murray, *J. Am. Chem. Soc.*, 1952, **74**, 1871.

7 D. H. Peterson, H. C. Murray, S. H. Eppstein, L. M. Reineke, A. Weintraub, P. D. Meister, and H. M. Leigh, *J. Am. Chem. Soc.*, 1952, **74**, 5933.

8 J. Fried, R. W. Thoma, and J. R. Gerke, *J. Am. Chem. Soc.*, 1952, **74**, 3692.

9 H. G. Davies, R. H. Green, D. R. Kelly, and S. M. Roberts, in 'Biotransformations in Preparative Organic Chemistry', London, 1989.

10 C. L. Smith, E. M. Hampton, J. A. Pederson, L. R. Pennington, and D. W. Bourne, *Pharmacotherapy*, 1994, **14**, 471.

11 M. Murray, *Int. J. Mol. Medicine*, 1999, **3**, 227.

12 H. J. M. van den Brink, R. F. M. van Gorcom, C. van den Hondel, and P. J. Punt, *Fungal Genetics and Biology*, 1998, **23**, 1.

13 B. P. Unger, I. C. Gunsalus, and S. G. Sligar, *J. Biol. Chem.*, 1986, **261**, 1158.

14 M. Katagiri, B. N. Ganguli, and I. C. Gunsalus, *J. Biol. Chem.*, 1968, **243**, 3543.

15 I. C. Gunsalus and S. G. Sligar, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1978, **47**, 1.

16 T. L. Poulos, B. C. Finzel, and A. J. Howard, *J. Mol. Biol.*, 1987, **195**, 687.

17 T. L. Poulos, B. C. Finzel, and A. J. Howard, *Biochemistry*, 1986, **25**, 5314.

18 T. L. Poulos, B. C. Finzel, I. C. Gunsalus, G. C. Wagner, and J. Kraut, *J. Biol. Chem.*, 1985, **260**, 16122.

19 S. G. Jezequel, *J. Mol. Catal. B-Enzymatic*, 1998, **5**, 371.

20 H. L. Holland, T. A. Morris, P. J. Nava, and M. Zabic, *Tetrahedron*, 1999, **55**, 7441.

21 G. J. Grogan and H. L. Holland, *J. Mol. Catal. B: Enzymatic*, 2000, **9**, 1.

22 A. Kergomard, M. F. Renard, and H. Veschambre, *J. Org. Chem.*, 1982, **47**, 792.

23 C. Fuganti, J. Minut, G. Pedrocchi-Fantoni, and S. Servi, *J. Mol. Catal. B: Enzymatic*, 1998, **4**, 47.

24 H. L. Holland and F. M. Brown, *Tetrahedron: Asymmetry*, 1998, **9**, 535.

25 H. L. Holland, P. R. Andreana, and F. M. Brown, *Tetrahedron: Asymmetry*, 1999, **10**, 2833.

26 A. Archelas, J. D. Fourneron, and R. Furstoss, *Tetrahedron Lett.*, 1988, **29**, 6611.

27 V. Gotor, M. Quiros, R. Liz, J. Frigola, and R. Fernandez, *Tetrahedron*, 1997, **53**, 6421.

28 B. Vigne, A. Archelas, and R. Furstoss, *Tetrahedron*, 1986, **42**, 2451.

29 B. Vigne, A. Archelas, and R. Furstoss, *Tetrahedron*, 1991, **47**, 1447.

30 J. Ilavsky and H. L. Herzog, in 'US Patent US 30139', 1961.

31 G. S. Fonken, M. E. Herr, H. C. Murray, and L. M. Reineke, *J. Am. Chem. Soc.*, 1967, **89**, 672.

32 V. Lamare, J. D. Fourneron, R. Furstoss, C. Ehret, and B. Corbier, *Tetrahedron Lett.*, 1987, **28**, 6269.

33 R. A. Johnson, H. C. Murray, L. M. Reineke, and G. S. Fonken, *J. Org. Chem.*, 1969, **34**, 2279.

34 R. A. Johnson, E. H. Milton, H. C. Murray, and G. S. Fonken, *J. Org. Chem.*, 1968, **33**, 3187.

35 C. R. Davis, R. A. Johnson, J. I. Ciadella, W. F. Liggett, S. A. Mizsak, and V. P. Marshall, *J. Org. Chem.*, 1997, **62**, 2244.

36 N. Floyd, F. Munyemana, S. M. Roberts, and A. J. Willetts, *J. Chem. Soc., Perkin Trans. 1*, 1993, 881.

37 R. Furstoss, A. Archelas, J. D. Fourneron, and B. Vigne, 'Enzymes as Catalysts in Organic Chemistry', ed. P. Schneider, 1986.

38 R. Furstoss, A. Archelas, B. Waegell, J. L. Petit, and L. Deveze, *Tetrahedron Lett.*, 1980, **21**, 451.

39 S. Pietz, D. Wolker, and G. Haufe, *Tetrahedron*, 1997, **53**, 17067.

40 A. Archelas, J. D. Fourneron, and R. Furstoss, *J. Org. Chem.*, 1988, **53**, 1797.

41 G. Braunegg, A. deRaadt, S. Feichtenhofer, H. Griengl, I. Kopper, A. Lehmann, and H. J. Weber, *Angew. Chem., Int. Ed. Engl.*, 1999, **38**, 2763.

42 A. M. Warhurst, K. F. Clarke, R. A. Hull, R. A. Holt, and C. A. Fewson, *Appl. Environ. Microbiol.*, 1994, **60**, 1137.

43 J. H. A. Apajahti and M. S. Salkinoja-Salonen, *Appl. Microbiol. Biotechnol.*, 1986, **25**, 62.

44 R. Valo and M. Salkinoja-Salonen, *Appl. Microbiol. Biotechnol.*, 1986, **25**, 68.

45 R. J. Valo, M. M. Haggblom, and M. S. Salkinoja-Salonen, *Water Research*, 1990, **24**, 253.

46 J. Sikkema and J. A. M. d. Bont, *Appl. Environ. Microbiol.*, 1993, **59**, 567.

47 G. J. MacMichael and L. R. Brown, *Appl. Environ. Microbiol.*, 1987, **53**, 65.

48 G. Cardini and P. Jurtshuk, *J. Biol. Chem.*, 1970, **245**, 2789.

49 K. Nakajima, A. Sato, Y. Takahara, and T. Iida, *Agric. Biol. Chem.*, 1985, **49**, 1993.

50 R. Hechtberger, G. Wirnsberger, M. Mischitz, N. Klempier, and K. Faber, *Tetrahedron: Asymmetry*, 1993, **4**, 1161.

51 M. Kobayashi, T. Nagasawa, and H. Yamada, *Trends Biotechnol.*, 1992, **10**, 402.

52 M. A. Cohen, J. S. Parratt, and N. J. Turner, *Tetrahedron: Asymmetry*, 1992, **3**, 1543.

53 T. M. Beard and M. I. Page, *Antonie Van Leeuwenhoek International J. Gen. Mol. Microbiol.*, 1998, **74**, 99.

54 C. T. Evans, S. M. Roberts, K. A. Shoberu, and A. G. Sutherland, *J. Chem. Soc., Perkin Trans. 1*, 1992, 589.

55 S. J. C. Taylor, R. McCague, R. Wisdom, C. Lee, K. Dickson, G. Ruecroft, F. O'Brien, J. Littlechild, J. Bevan, S. M. Roberts, and C. T. Evans, *Tetrahedron: Asymmetry*, 1993, **4**, 117.

56 M. Chartrain, B. Jackey, C. Taylor, V. Sandford, K. Gbewonyo, L. Lister, L. Dimichele, C. Hirsch, B. Heimbuch, C. Maxwell, D. Pascoe, B. Buckland, and R. Greasham, *J. Fermentation Bioeng.*, 1998, **86**, 550.

57 D. P. O'Keefe and P. A. Harder, *Mol. Microbiol.*, 1991, **5**, 2099.

58 J. B. Sutherland, J. P. Freeman, A. L. Selby, P. P. Fu, D. W. Miller, and C. E. Cerniglia, *Arch. Microbiol.*, 1990, **154**, 260.

59 J. B. Sutherland, *Appl. Environ. Microbiol.*, 1986, **52**, 98.

60 M. K. Trower, F. S. Sariaslani, and D. P. O'Keefe, *J. Bacteriol.*, 1989, **171**, 1781.

61 I. Nagy, F. Compernolle, K. Ghys, J. Vanderleyden, and R. d. Mot, *Appl. Environ. Microbiol.*, 1995, **61**, 2056.

62 I. Nagy, G. Schoofs, F. Compernolle, P. Proost, J. Vanderleyden, and R. d. Mot, *J. Bacteriol.*, 1995, **177**, 676.

63 H. Ohta, S. Matsumoto, Y. Okanoto, and T. Sugai, *Chem. Lett.*, 1989, 625.

64 B. Angelova, S. Mutafov, T. Avramora, and L. Boyadjieva, *Process Biochem.*, 1996, **31**, 179.

65 G. Cardini and P. Jurtshuk, *J. Biol. Chem.*, 1968, **243**.

66 P. J. Chapman, J. F. Kuo, and I. C. Gunsalus, *Fed. Proc.*, 1963, **22**, 296.

67 P. J. Chapman, G. Meerman, and I. C. Gunsalus, *Biochem. Biophys. Res. Commun.*, 1965, **20**, 104.

68 G. Grogan, S. L. Flitsch, and N. J. Turner, *Unpublished results.*

69 F. S. Sariaslani, L. R. McGee, M. K. Trower, and F. G. Kitson, *Biochem. Biophys. Res. Commun.*, 1990, **170**, 456.

70 M. K. Trower, F. S. Sariaslani, and F. G. Kitson, *Biochem. Biophys. Res. Commun.*, 1988, **157**, 1417.

71 U. Karlson, D. F. Dwyer, S. W. Hooper, E. R. B. Moore, K. N. Timmis, and L. D. Eltis, *J. Bacteriol.*, 1993, **175**, 1467.

72 L. D. Eltis, U. Karlson, and K. N. Timmis, *Eur. J. Biochem.*, 1993, **213**, 211.

73 S. J. Aitken, G. Grogan, C. S. Y. Chow, N. J. Turner, and S. L. Flitsch, *J. Chem. Soc., Perkin Trans 1*, 1998, 3365.

74 S. J. Aitken, 'Cytochrome P-450 Monooxygenases: Their Application in Biocatalysis', MSc (by Research), University of Edinburgh, 1997.

75 J. D. Fourneron, A. Archelas, B. Vigne, and R. Furstoss, *Tetrahedron*, 1987, **43**, 2273.

76 J. T. Groves, G. A. McClusky, R. E. White, and M. J. Coon, *Biochem. Biophys. Res. Commun.*, 1978, **81**, 154.

77 J. D. Fourneron, A. Archelas, and R. Furstoss, *J. Org. Chem.*, 1989, **54**, 2478.

78 Y. L. Chow, C. J. Colon, and J. N. S. Tam, *Can. J. Chem*, 1968, **46**, 2822.

79 R. A. Johnson, *J. Org. Chem.*, 1968, **33**, 3627.

80 W. Carruthers, J. D. Prail, S. M. Roberts, and A. J. Willetts, *J. Chem. Soc., Perkin Trans. 1*, 1990, 2854.

81 R. A. Johnson, M. E. Herr, H. C. Murray, C. G. Chidester, and F. Han, *J. Org. Chem.*, 1992, **57**, 7209.

82 H. L. Holland, M. Conn, P. C. Chenchaiah, and F. M. Brown, *Tetrahedron Lett.*, 1988, **29**, 6393.

83 M. E. Herr, R. A. Johnson, H. C. Murray, L. M. Reineke, and G. S. Fonken, *J. Org. Chem.*, 1968, **33**, 3201.

84 C. R. Davis, R. A. Johnson, J. I. Ciadella, W. F. Liggett, S. A. Mizsak, F. Han, and V. P. Marshall, *J. Org. Chem.*, 1997, **62**, 2252.

85 A. de Raadt, H. Griengl, M. Petsch, P. Plachato, N. Schoo, H. Weber, G. Braunegg, I. Kopper, M. Kreiner, and A. Zeiser, *Tetrahedron: Asymmetry*, 1996, **7**, 473.

86 A. de Raadt, H. Griengl, M. Petsch, P. Plachato, N. Schoo, H. Weber, G. Braunegg, I. Kopper, M. Kreiner, and A. Zeiser, *Tetrahedron: Asymmetry*, 1996, **7**, 491.

87 A. de Raadt, H. Griengl, M. Petsch, P. Plachato, N. Schoo, H. Weber, G. Braunegg, I. Kopper, M. Kreiner, A. Zeiser, and K. Kieslich, *Tetrahedron: Asymmetry*, 1996, **7**, 467.

88 D. A. Grayson, Y. B. Tewari, M. P. Mayhew, V. L. Vilker, and R. N. Goldberg, *Arch. Biochem. Biophys.*, 1996, **332**, 239.

89 T. L. Poulos and A. J. Howard, *Biochemistry*, 1987, **26**, 8165.

90 K. Gill, J. F. Menez, D. Lucas, and R. A. Dietrich, *Alcoholism: Clinical and Experimental Research*, 1992, **16**, 910.

91 M. Sharrock, E. Munck, P. G. Brunner, V. Marshall, J. B. Lipscomb, and I. C. Gunsalus, *Biochemistry*, 1973, **12**, 258.

92 S. M. Fowler, P. A. England, A. C. G. Westlake, D. Rouch, D. P. Nickerson, C. Blunt, D. Braybrook, S. West, L. L. Wong, and S. L. Flitsch, *J. Chem. Soc., Chem. Commun.*, 1994, 2761.

93 J. P. Jones, E. J. O'Hare, and L. L. Wong, *J. Chem. Soc., Chem. Commun.*, 2000, 247.

94 A. Schmid, A. Kollmer, B. Sonnleitner, and B. Witholt, *Bioprocess Eng.*, 1999, **20**, 91.

95 S. L. Flitsch, S. J. Aitken, C. S. Y. Chow, G. Grogan, and A. Staines, *Bioorg. Chem.*, 1999, **27**, 81.

96 C. S.-Y. Chow, 'Selective hydroxylations catalysed by cytochrome P-450 monooxygenases', PhD Thesis, University of Edinburgh, 1998.

97 F. Mohamadi, N. G. J. Richards, W. C. Guids, R. Liskamp, M. Lipton, C. Caufield, G. Chang, T. Hendrickson, and W. C. Still, *J. Comput. Chem.*, 1990, **11**, 440.

98 N. L. Allinger, *J. Am. Chem. Soc.*, 1977, **99**, 8127.

99 W. C. Still, A. Tempczyk, R. C. Hawley, and T. Hendrickson, *J. Am. Chem. Soc.*, 1990, **112**, 6127.

100 G. Chang, W. C. Guida, and W. C. Still, *J. Am. Chem. Soc.*, 1989, **111**, 4379.

101 H. L. Holland, M. Kindermann, S. Kumaresan, and T. Stefanac, *Tetrahedron: Asymmetry*, 1993, **4**, 1353.

102 J. A. Dale, D. L. Dull, and H. S. Mosher, *J. Org. Chem.*, 1969, **34**, 2543.

103 J. A. Dale and H. S. Mosher, *J. Am. Chem. Soc.*, 1973, **95**, 512.

104 G. R. Sullivan, J. A. Dale, and H. S. Mosher, *J. Org. Chem.*, 1973, **38**, 2143.

105 F. Yasuhara, S. Yamaguchi, M. Takeda, T. Abe, and S. Miyano, *Bull. Chem. Soc. Jpn.*, 1991, **64**, 3390.

106 K. Mori, A. Horinaka, and M. Kido, *Liebigs Ann. Chem.*, 1994, 817.

107 S. Yao, M. Robertson, F. Reichel, R. G. Hazell, and K. A. Jorgensen, *J. Org. Chem.*, 1999, **64**, 6677.

108 D. Parker, R. J. Taylor, G. Ferguson, and A. Tonge, *Tetrahedron*, 1986, **42**, 617.

109 H. Gerlach and B. Zagalak, *J. Chem. Soc., Chem. Commun.*, 1973, 274.

110 P. Darrigo, G. Pedrochi-Fantoni, and S. Servi, *Adv. Appl. Microbiol.*, 1997, **44**, 81.

111 Z. Li, H. J. Feiten, J. B. van Beilen, W. Duetz, and B. Witholt, *Tetrahedron: Asymmetry*, 1999, **10**, 1323.

112 E. L. White, N. B. Barker, L. J. Macy, S. C. Shaddix, C. McCaleb, J. A. S. III, R. Vince, and W. M. Shannon, *Biochem. Biophys. Res. Commun.*, 1989, **161**, 393.

113 H. F. Olivo and J. Yu, *J. Chem. Soc., Perkin Trans. 1*, 1998, 391.

114 D. J. Leak, S. L. R. Ellison, C. Murricane, and P. B. Baker, *Biocatalysis*, 1988, **1**, 197.

115 C. S. Cooper, P. L. Klock, D. T. W. Chu, D. J. Hardy, R. N. Swanson, and J. J. Plattner, *J. Med. Chem.*, 1992, **35**, 1392.

116 L. Alig, A. Edenhoffer, P. Hadvary, M. Huerzeler, D. Knopp, N. Muller, B. Steiner, A. Trzeciak, and T. Weller, *J. Med. Chem.*, 1992, **35**, 4393.

117 S. Hoyer and P. Laszlo, *Synthesis*, 1986, 656.

118 S. Koto, N. Morishima, R. Kawahara, K. Ishikawa, and S. Zen, *Bull. Chem. Soc. Jpn.*, 1982, **55**, 1092.

119 H. Kurokawa, T. Ishigami, K. Morimoto, and A. Siegimori, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 959.

120 J. F. Garst and C. D. Smith, *J. Am. Chem. Soc.*, 1976, **98**, 1526.

121 J. Schulz and D. Gani, *J. Chem. Soc., Perkin Trans. 1*, 1997, 657.

122 F. Calvani, P. Crotti, C. Gardelli, and M. Pineschi, *Tetrahedron*, 1994, **50**, 12999.

123 M. Chini, P. Crotti, L. A. Flippin, and F. Macchia, *J. Org. Chem.*, 1990, **55**, 4265.

124 A. M. Warhurst and C. A. Fewson, *Crit. Rev. Biotechnol.*, 1994, **14**, 29.

125 J. Ooyama and K. Yamada, *Antonie Van Leeuwenhoek International J. Gen. Mol. Microbiol.*, 1965, **31**, 45.

126 S. H. El-Sharkawy, W. Yang, L. Dostal and J. P. N. Rosazza, *App. Environ. Microbiol*, 1992, **58**, 2116.

7 Appendix I

A. CRYSTAL DATA

Empirical formula	C20 H26 N O8	
Formula weight	408.42	
Wavelength	0.71073 Å	
Temperature	150(2) K	
Crystal system	Monoclinic	
Space group	P21	
Unit cell dimensions	a = 11.423(5) Å	$\alpha = 90°$
	b = 6.727(3) Å	$\beta = 105.732(8)°$
	c = 13.889(6) Å	$\gamma = 90°$
Volume	1027.3(8) $Å^3$	
Number of reflections for cell	515 ($2.5 < \theta < 19.5°$)	
Z	2	
Density (calculated)	1.320 Mg/m^3	
Absorption coefficient	0.102 mm^{-1}	
F(000)	434	

B. DATA COLLECTION

Crystal description	Colourless needle
Crystal size	0.26 x 0.02 x 0.02 mm
θ range for data collection	1.52 to 25.01°
Index ranges	-13<=h<=13, -7<=k<=8, -13<=l<=16
Reflections collected	5229
Independent reflections	3161 [R(int) = 0.0520]
Scan type	Omega

Absorption correction	Sadabs
	(T_{min}= 0.588, T_{max}=1.000)

C. SOLUTION AND REFINEMENT

Solution	direct (SHELXS-97 (Sheldrick, 1990))
Refinement type	Full-matrix least-squares on F^2
Program used for refinement	SHELXL-97
Hydrogen atom placement	geometric
Hydrogen atom treatment	riding
Data / restraints / parameters	3161/1/272
Goodness-of-fit on F^2	0.871
Conventional R [F>4σ(F)]	R1 = 0.0571 [2003 data]
Weighted R (F^2 and all data)	wR2 = 0.1340
Absolute structure parameter	-0.1(18)
Extinction coefficient	0.014(3)
Final maximum delta/sigma	0.001
Weighting scheme	calc w=1/[\s^2^(Fo^2)+(0.0737P)^2+0.0000P] where P=(Fo^2+2Fc^2)/3
Largest diff. peak and hole	0.272 and -0.247 e. Å^{-3}

Table 1. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (A^2 x 10^3). U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

Atom	x	y	z	U(eq)
O(1)	1643(2)	6139(4)	3136(2)	39(1)
C(2)	2700(3)	4890(7)	3658(3)	31(1)
C(3)	3783(4)	6341(7)	3902(3)	38(1)
C(4)	3486(4)	7650(7)	4710(3)	38(1)
C(5)	2289(3)	6804(7)	4842(3)	31(1)
O(6)	572(3)	8516(6)	3651(2)	50(1)
C(6)	1375(4)	7309(7)	3847(3)	35(1)
C(7)	2416(3)	4569(7)	4676(3)	31(1)
O(8)	3543(3)	1828(5)	3395(2)	48(1)
C(8)	2848(4)	3167(8)	3056(3)	38(1)
O(9)	2204(2)	3259(5)	2091(2)	42(1)
C(10)	2487(4)	1727(7)	1435(3)	42(1)
C(11)	2094(4)	2573(7)	367(3)	45(1)
C(12)	1314(4)	935(8)	-212(3)	45(1)
O(13)	776(3)	-11(5)	465(2)	50(1)
C(14)	1733(4)	-120(8)	1402(3)	50(1)
O(15)	2067(3)	-343(5)	-573(2)	46(1)
C(16)	1405(4)	-1928(7)	-1182(3)	51(1)
C(17)	2238(4)	-3472(7)	-1367(3)	39(1)
C(18)	1738(4)	-5111(8)	-1937(3)	42(1)
C(19)	2444(4)	-6642(9)	-2113(3)	49(1)
C(20)	3705(4)	-6553(9)	-1701(3)	45(1)
C(21)	4226(4)	-4918(8)	-1144(3)	49(1)
C(22)	3499(4)	-3399(8)	-968(3)	46(1)
N(23)	4464(5)	-8150(8)	-1885(3)	59(1)

O(24)	5570(4)	-8024(7)	-1510(3)	86(1)
O(25)	3987(4)	-9563(6)	-2413(3)	70(1)
C(51)	1931(4)	7508(7)	5760(3)	44(1)
C(71)	1258(3)	3381(8)	4581(3)	42(1)
C(72)	3454(4)	3589(8)	5483(3)	40(1)

Table 2. Bond lengths [Å] and angles [deg].

O(1)-C(6)	1.361(5)
O(1)-C(2)	1.488(4)
C(2)-C(8)	1.466(6)
C(2)-C(3)	1.540(6)
C(2)-C(7)	1.549(5)
C(3)-C(4)	1.534(6)
C(4)-C(5)	1.537(6)
C(5)-C(51)	1.518(6)
C(5)-C(6)	1.527(5)
C(5)-C(7)	1.534(6)
O(6)-C(6)	1.200(5)
C(7)-C(71)	1.520(6)
C(7)-C(72)	1.541(5)
O(8)-C(8)	1.209(5)
C(8)-O(9)	1.344(5)
O(9)-C(10)	1.468(5)
C(10)-C(14)	1.506(7)
C(10)-C(11)	1.538(6)
C(11)-C(12)	1.505(6)
C(12)-O(15)	1.401(5)
C(12)-O(13)	1.409(5)
O(13)-C(14)	1.457(5)
O(15)-C(16)	1.442(5)
C(16)-C(17)	1.478(6)
C(17)-C(18)	1.387(6)
C(17)-C(22)	1.397(6)
C(18)-C(19)	1.371(7)
C(19)-C(20)	1.399(6)

C(20)-C(21)	1.383(7)
C(20)-N(23)	1.447(6)
C(21)-C(22)	1.380(6)
N(23)-O(24)	1.232(5)
N(23)-O(25)	1.234(5)

C(6)-O(1)-C(2)	106.6(3)
C(8)-C(2)-O(1)	111.9(3)
C(8)-C(2)-C(3)	114.6(3)
O(1)-C(2)-C(3)	104.3(3)
C(8)-C(2)-C(7)	119.7(4)
O(1)-C(2)-C(7)	100.4(3)
C(3)-C(2)-C(7)	104.0(3)
C(4)-C(3)-C(2)	101.6(3)
C(3)-C(4)-C(5)	104.7(3)
C(51)-C(5)-C(6)	114.7(3)
C(51)-C(5)-C(7)	119.3(3)
C(6)-C(5)-C(7)	98.7(3)
C(51)-C(5)-C(4)	115.8(4)
C(6)-C(5)-C(4)	102.8(3)
C(7)-C(5)-C(4)	102.8(3)
O(6)-C(6)-O(1)	122.3(4)
O(6)-C(6)-C(5)	130.5(4)
O(1)-C(6)-C(5)	107.1(4)
C(71)-C(7)-C(5)	114.4(3)
C(71)-C(7)-C(72)	108.6(4)
C(5)-C(7)-C(72)	113.3(3)
C(71)-C(7)-C(2)	112.7(3)
C(5)-C(7)-C(2)	93.0(3)
C(72)-C(7)-C(2)	114.4(3)

O(8)-C(8)-O(9)	123.2(4)
O(8)-C(8)-C(2)	122.2(4)
O(9)-C(8)-C(2)	114.6(4)
C(8)-O(9)-C(10)	115.7(3)
O(9)-C(10)-C(14)	112.2(3)
O(9)-C(10)-C(11)	106.5(4)
C(14)-C(10)-C(11)	105.1(4)
C(12)-C(11)-C(10)	102.8(4)
O(15)-C(12)-O(13)	112.6(4)
O(15)-C(12)-C(11)	108.0(4)
O(13)-C(12)-C(11)	106.0(3)
C(12)-O(13)-C(14)	105.1(3)
O(13)-C(14)-C(10)	105.7(4)
C(12)-O(15)-C(16)	112.8(3)
O(15)-C(16)-C(17)	111.2(4)
C(18)-C(17)-C(22)	118.4(5)
C(18)-C(17)-C(16)	118.2(4)
C(22)-C(17)-C(16)	123.4(4)
C(19)-C(18)-C(17)	121.8(4)
C(18)-C(19)-C(20)	119.0(5)
C(21)-C(20)-C(19)	120.1(5)
C(21)-C(20)-N(23)	120.1(4)
C(19)-C(20)-N(23)	119.7(5)
C(22)-C(21)-C(20)	120.0(4)
C(21)-C(22)-C(17)	120.6(4)
O(24)-N(23)-O(25)	122.8(5)
O(24)-N(23)-C(20)	118.0(5)
O(25)-N(23)-C(20)	119.2(5)

Symmetry transformations used to generate equivalent atoms

Table 3. Anisotropic displacement parameters (A^2 x 10^3). The anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2 a^{*2} U11 + ... + 2 h k a^* b^* U12]$

	U11	U22	U33	U23	U13	U12
O(1)	33(2)	50(2)	27(2)	7(2)	-4(1)	11(2)
C(2)	23(2)	38(3)	28(2)	11(2)	1(2)	7(2)
C(3)	30(2)	46(3)	36(2)	3(2)	6(2)	-6(2)
C(4)	30(2)	43(3)	33(2)	2(2)	-2(2)	5(2)
C(5)	28(2)	40(3)	20(2)	10(2)	-3(2)	9(2)
O(6)	40(2)	62(2)	39(2)	5(2)	-5(2)	19(2)
C(6)	30(3)	38(3)	31(2)	4(2)	-1(2)	5(2)
C(7)	21(2)	42(3)	27(2)	6(2)	0(2)	0(2)
O(8)	55(2)	48(2)	32(2)	-1(2)	-1(2)	15(2)
C(8)	39(3)	43(3)	29(2)	1(2)	7(2)	-5(3)
O(9)	40(2)	57(2)	23(2)	-3(2)	-3(1)	6(2)
C(10)	41(3)	54(3)	28(2)	0(3)	4(2)	11(3)
C(11)	54(3)	48(3)	30(2)	5(2)	7(2)	1(3)
C(12)	50(3)	57(3)	23(2)	-2(2)	5(2)	-4(3)
O(13)	45(2)	67(2)	31(2)	-3(2)	1(2)	-8(2)
C(14)	65(3)	56(3)	27(2)	2(2)	9(2)	4(3)
O(15)	46(2)	58(2)	29(2)	-7(2)	3(1)	-5(2)
C(16)	56(3)	52(4)	33(3)	-8(3)	-7(2)	-13(3)
C(17)	44(3)	43(3)	24(2)	-3(2)	1(2)	-8(3)
C(18)	42(3)	53(3)	26(2)	-3(2)	1(2)	-5(3)
C(19)	57(3)	64(4)	18(2)	-6(2)	-1(2)	-17(3)
C(20)	52(3)	60(4)	22(2)	2(3)	6(2)	-4(3)
C(21)	41(3)	70(4)	30(2)	1(3)	3(2)	-6(3)
C(22)	48(3)	57(4)	28(2)	-6(2)	5(2)	-10(3)

N(23)	76(3)	69(4)	30(2)	4(2)	13(2)	12(3)
O(24)	63(2)	132(4)	54(2)	-11(2)	4(2)	26(3)
O(25)	90(3)	59(3)	60(3)	-9(2)	17(2)	3(2)
C(51)	39(3)	55(3)	33(2)	5(2)	0(2)	13(2)
C(71)	35(2)	48(3)	40(3)	7(2)	8(2)	-2(2)
C(72)	40(2)	49(3)	27(2)	8(2)	1(2)	7(2)

Table 4. Hydrogen coordinates (x 10^4) and isotropic displacement parameters (A^2 x 10^3).

	x	y	z	U(eq)
H(3A)	4564	5631	4162	46
H(3B)	3814	7132	3308	46
H(4A)	3384	9055	4492	45
H(4B)	4141	7568	5344	45
H(10)	3375	1404	1635	51
H(11A)	1623	3816	340	54
H(11B)	2806	2834	108	54
H(12)	671	1508	-783	53
H(14A)	2236	-1325	1418	60
H(14B)	1380	-159	1979	60
H(16A)	920	-1380	-1829	61
H(16B)	835	-2533	-841	61
H(18)	881	-5174	-2213	50
H(19)	2083	-7748	-2509	58
H(21)	5084	-4842	-882	58
H(22)	3859	-2293	-572	55
H(51A)	2560	7120	6364	66
H(51B)	1154	6901	5771	66
H(51C)	1847	8959	5740	66
H(71A)	1415	1973	4485	62
H(71B)	621	3866	4005	62
H(71C)	992	3540	5190	62
H(72A)	3491	2170	5335	60
H(72B)	3303	3753	6140	60
H(72C)	4228	4221	5486	60

Biohydroxylations of Cbz-protected alkyl substituted piperidines by *Beauveria bassiana* ATCC 7159

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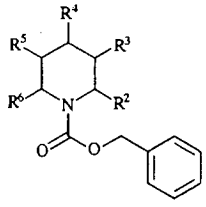
N-Benzyloxycarbonyl (Cbz) protected piperidines are hydroxylated with greater regioselectivity than the corresponding *N*-benzoyl analogues when incubated with the fungus *Beauveria bassiana* ATCC 7159. Cbz-protected piperidines **1–3**, **5–7**, have been biotransformed by growing cell suspensions of this fungus to yield predominantly 4-hydroxylated products in up to 48% yield. The regiospecificity of hydroxylation was only compromised significantly with *N*-Cbz-3-methylpiperidine **3** and *N*-Cbz-2-methylpiperidine **4** where hydroxylation occurred in both the 3 and 4 positions.

Introduction

The hydroxylation of non-activated carbon centres remains a challenge in organic synthesis. In most cases, a biocatalytic route is favoured, most notably in the industrial conversion of steroids by fungi.[1] Such biocatalytic methods are preferred for reasons of both regio- and stereoselectivity and the technique of fungal biohydroxylation has now been applied to the production of a wide range of hydroxylated derivatives of xenobiotic compounds. Most notable of these is the use of the entomopathogen *Beauveria bassiana* to selectively hydroxylate a wide range of organic compounds including N-containing heterocycles.[2] Pioneering work by the group of Fonken and Johnson at the Upjohn company established that the biohydroxylation of N-heterocycles was much improved by their conjugation to an aromatic moiety linked to the heterocycle *via* a bridge with an electron rich centre.[3] Detailed studies revealed that *Sporotrichum sulfurescens* ATCC 7159, now redesignated *Beauveria bassiana*, would selectively hydroxylate *N*-benzoylalkylpiperidines to give optically active hydroxypiperidines.[4] Hydroxypiperidines are constituents of many pharmacologically and biochemically important natural products including pseudoconhydrine[5] and esters of 1-methylpiperidin-3-ol[6] which have cholinergic or anticholinergic properties.

A particular feature of biohydroxylations reactions is the possible formation of a large number of reaction products given that the reaction is not generally directed by a particular functional group. For example, *N*-benzoylpiperidine studied by Johnson can potentially lead to six different hydroxylation products, and the number increases dramatically with substitution within the piperidine ring. It is therefore of great interest to understand how to direct regio- and stereoselectivity of hydroxylation to give the desired alcohol. Changes in selectivity can either be achieved by using a different biocatalyst or by temporarily changing the structural characteristics of the substrate using protecting groups.[7] Here we show that the selectivity of biohydroxylation by *Beauveria bassiana* can be modified by using benzyloxycarbonyl (Cbz) instead of benzoyl protected piperidines. The Cbz-group has the additional advantage that it is more amenable to facile removal than a benzoyl group.[8] A number of Cbz-protected alkylpiperidines **1–8** were therefore synthesised and incubated with *Beauveria bassiana* ATCC 7159 and the results of biotransformation compared with those obtained with the corresponding *N*-benzoyl analogues.



1 R = H

2 R^n = H, R^4 = Me

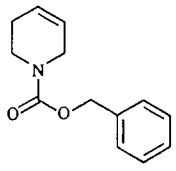
3 R^n = H, R^3 = Me

4 R^n = H, R^2 = Me

5 R^n = H, R^2, R^6 = Me

6 R^n = H, R^2 = Et

7 R^n = H, R^3, R^3 = Me

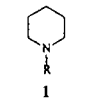
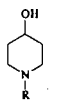
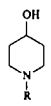
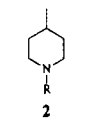
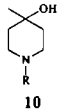
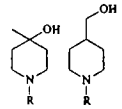
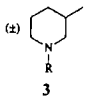
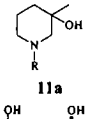
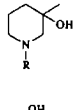
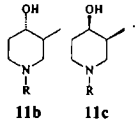
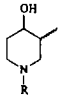
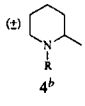
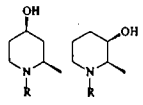
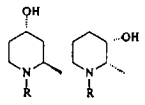
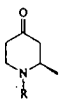
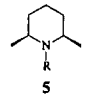
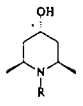
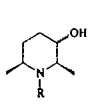
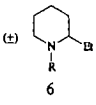
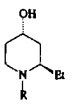
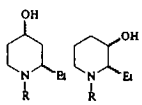
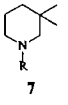
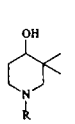
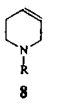


8

Results and discussion

Cbz-protected alkylpiperidines **1–8** were synthesised from the unprotected piperidines by treatment with benzyl chloroformate in the presence of potassium carbonate. The results of the biohydroxylations are summarised in Table 1, including a comparison with those obtained using the *N*-benzoyl analogues, as reported previously.[4] The unsubstituted Cbz-protected piperidine **1** is hydroxylated almost exclusively at the 4-position to give **9** (33% yield), in common with results obtained with *N*-benzoyl[4] and *N*-phenyl[9] derivatives. The structure was easily assigned from inspection of the ^{13}C NMR spectrum which clearly showed no loss of symmetry in the hydroxylated product. This selectivity was completely conserved when the 4-methylpiperidine derivative **2** was the substrate, with hydroxylation occurring at the tertiary carbon to give **10** (45% yield). This is not surprising as the radical mechanism by which hydroxylation by *Beauveria bassiana* is proposed to occur[10] would favour this product due to the stability of the tertiary radical intermediate. It is interesting to note that biohydroxylation of the *N*-benzoyl derivative[4] resulted in a 1:2 mixture of the 4-hydroxy compound and the 4-hydroxymethyl compound as a result of competing hydroxylation of the C-4 methyl group.

Table 1 Comparison of biohydroxylation of *N*-benzoyl and *N*-Cbz protected alkylpiperidines by *Beauveria bassiana* ATCC 7159

Substrate	Combined yield[a] Hydroxylated products (%) R = $PhCH_2OCO$	Major products R = $PhCH_2OCO$	Major products R = PhCO (data taken from ref. 4)
 **1**	33	 **9**	
 **2**	45	 **10**	
(±)  **3**	24	 **11a**	
		 **11b** **11c**	
(±)  4^b	14	 **12a** **12b**	 
 **5**	5	 **13**	
(±)  **6**	45	 **14**	
 **7**	48	 **15**	–
 **8**	0	–	–

[a] Combined yields calculated prior to separation. [b] Preparation of substrates **4** and **8** where R = Cbz have been reported previously.[21,22]

The insertion of an O-CH_2 into the protecting group appears to have improved the regioselectivity of hydroxylation.

Three products (**11a–c**) were isolated from the transformation of *N*-benzyloxycarbonyl-3-methylpiperidine **3**, of which **11a** and **11c** were very difficult to separate by either chromatography on silica or preparative HPLC. The mixture of **11a** and **11c** was therefore subjected to acetylation conditions in an attempt to ease separation. Only one of the two products was acetylated (as shown by NMR and mass spectrometry) resulting in a mixture of **11a** and *O*-acetylated **11c**, which were then easily separated by preparative HPLC.

Product **11a** was found to be unaltered by the acetylation conditions. Mass spectrometry (atmospheric pressure chemical ionisation) showed an $(M + 1)^+$ peak at 250.1, confirming that **11a** is a monohydroxylated product. Since the characteristic C*H*OH signal was missing in the NMR spectrum, **11a** was assigned as the 3-methyl-3-hydroxy derivative.

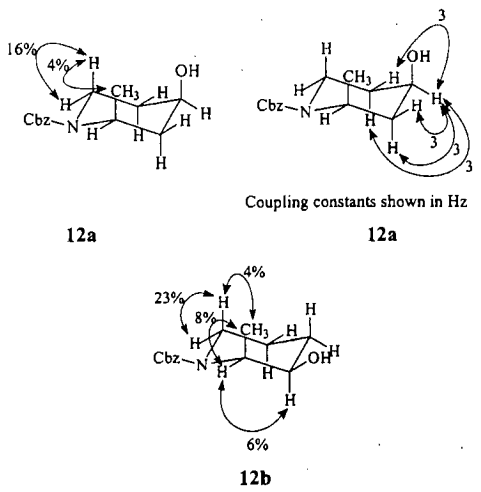
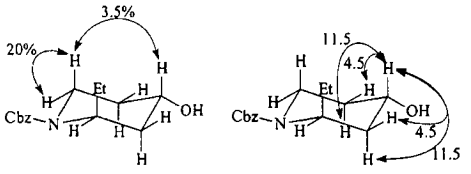


Fig. 1 NOE enhancements and coupling constants used in assigning hydroxylated products **12a** and **12b**.

The structure of **11c** was confirmed by NMR spectroscopy of the *O*-acetylated derivative. From the coupling pattern, it was possible to deduce that the hydroxy moiety was introduced in the axial position since the adjacent proton signal showed only three small couplings (*J* 3, 5 and 5 Hz). It was however not possible to establish the relative position of the methyl group from simple decoupling experiments since the C3-H signal is not easily identifiable. It is thought however that the methyl is most likely to lie in the equatorial position since a 3,4-diaxial conformation would be disfavoured and thus prone to ring-flip to give the 3,4-diequatorial conformation.

Product **11b** was identified by NMR spectroscopy as the isomer of **11c**, the 4-hydroxy-3-methyl derivative with the hydroxy group located equatorially. The signal due to the C-4 proton showed two large and one small couplings (*J* 3, 12, and 14 Hz) implying that the methyl group also adopts an equatorial position. The regioselectivity obtained in the biohydroxylation of **3** is the same as that observed for the *N*-benzoylpiperidine.[4] Evidently in this case, alignment of the piperidine ring in the enzyme active site is a more significant factor for hydroxylation regioselectivity than ease of radical formation which would favour formation of **11a**. When the fungus was challenged with the 2-methylpiperidine derivative **4** as substrate, equal amounts of products hydroxylated in the 3 (**12b**) and 4 (**12a**) positions but no 2-hydroxylated products were obtained. The structures of **12a** and **12b** were confirmed by NOE experiments, as shown in Fig. 1. The preference of 2-alkyl substituents for axial rather than equatorial positions is well documented in alkylpiperidine amides.[11,12] These conformational effects favour the 2,4-diaxial chair over the diequatorial conformation of **12a**.

One major concern about employing this series of substrates with a whole cell biotransformation system was the potential lability of the Cbz-protecting group to other enzymes in the fungus. Microbial carbamate hydrolases have been reported[13] and degradation of alkyl substituted aromatics by benzylic hydroxylation has been well studied.[14] It had also been reported that the inclusion of inverted urethane-type linkers in hydroxylation substrates for this fungus led to no biotransformation whatsoever.[15] In only one case were these possibilities implicated by results. When *N*-benzyloxycarbonyl-*cis*-2,6-dimethylpiperidine **5** (270 mg) was incubated with *Beauveria bassiana* only 10 mg of hydroxylated product was recovered. ^1H and ^{13}C NMR indicated this compound to once again be the 4-hydroxy



14 **14**

Fig. 2 NOE enhancements and coupling constants used in assigning 4-hydroxylated product **14** of biotransformation of **6**.

derivative **13**. However, a large amount of benzoic acid (92 mg) was also recovered from the reaction mixture suggesting a degradative mechanism involving a benzylic hydroxylase as has been previously described.[14] This degradation pathway is not relevant in the *N*-benzoyl analogues which is reflected in the better yield reported.

Although no active cell-free extract, nor pure enzyme work has been successfully conducted using preparations from *Beauveria bassiana* ATCC 7159, the results of the numerous whole-cell studies have been used to construct a crude model for the active site of what is assumed to be the sole hydroxylating species in this fungus.[15,16] This model suggests an optimum distance from the electron-rich site of attachment to the site of hydroxylation of between 3.3 and 6.2 Å.[16] The results with *N*-benzyloxycarbonylalkylpiperidines suggest that the active site of the *Beauveria* hydroxylating system is very sensitive to relatively small changes in the nature of the protecting group. The hydroxylase is clearly now disposed to hydroxylation in the 4-position, except where there is a 2- or 3-methyl substituent, when the selectivity is compromised. The distance from the carbonyl group to the putative site of hydroxylation is, of course, unchanged from *N*-benzoyl to *N*-benzyloxycarbonyl substrate. The results presented herein would suggest that the more critical characteristic for selective hydroxylation is the distance from carbonyl to an aromatic binding pocket. In addition, greater flexibility of the *N*-benzyloxycarbonyl molecule is afforded by rotation around the benzylic centre. This would appear to allow improved accommodation by the active site and therefore greater regioselectivity for some substrates.

The change in regioselectivity of hydroxylation around the piperidine ring from the 4- to the 3-position with regioisomeric alkyl substitution is difficult to rationalise in terms of steric restrictions in the active site of the hydroxylase. For piperidines **1** and **2** access to the favoured 4-position by the hydroxylating species is unrestricted, but methyl substitution at the 2- and 3-position must move the 4-position sufficiently far from this species that hydroxylation also occurs at the 3-position. One would expect that an extension to the 2-alkyl chain would therefore result in even more pronounced selectivity for the 3-position. However, when *N*-benzyloxycarbonyl-2-ethylpiperidine **6** is the substrate, hydroxylation occurs almost exclusively at the 4-position to give the 4-hydroxypiperidine derivative **14** (Fig. 2) in good yield.

In Johnson's work,[4] the relative position of the hydroxy group in the hydroxylated products from the 2-alkylpiperidines was assigned by analogy to the products of the 2-ethyl transformation and it has been well reported that, in rigid systems, the hydroxy group is introduced in a *trans* orientation with respect to the amide functional group.[2a] By the use of NMR analysis we have established that the hydroxy group is also introduced into the equatorial position in most cases, with the exceptions of the 2-methyl-4-hydroxy product **12a** where the OH was in an axial position (this was shown by the lack of an NOE enhancement between the axial C2-H and the C4-H and also from the coupling constants of the C*H*OH signal, showing no axial–axial coupling) and the 3-methyl-4-hydroxy product (the acetylated derivative of which was isolated) **11c** where the C*H*OAc signal showed no diaxial couplings.

It is interesting to note that Johnson and co-workers observed some enantiodifferentiation by the *Beauveria* hydroxylating system when operating on *N*-benzoalkylpiperidines.[4] The optical purity of our products has not been determined but it was seen that in all cases, a 72 hour incubation of the substrate with the fungus resulted in complete substrate disappearance implying that no substrate enantiomer (where relevant) is preferred to any great extent. It is likely, on the basis of previous work,[7] that the enantioselectivity and resulting enantiomeric excess (if relevant) will depend on many factors, including the time course of the reaction. There could be preferential hydroxylation of one enantiomer of the substrate at a single position as well as preferential metabolism of one of the product molecules, both of these examples would effect the enantiomeric excess of the identified product. An investigation into these factors is currently underway in our laboratories and will be published at a later date. Since the yield of hydroxylated products was approximately 50% it is unlikely that a resolution process is operating.

Two further substrates have been tested with the fungus which do not correlate with the substrate used by Fonken but add to the overall series and the investigation of the tolerance of the active site to changes in the substrate. It was found on incubation of the 3,3-dimethylpiperidine derivative **7** with the fungus that the 4-hydroxylated product **15** was produced. It was also noted that the rate of this hydroxylation was enhanced and that no substrate remained after two days, whereas other reactions required three days for complete substrate disappearance. This is likely to be a consequence of the reaction being directed by the two methyl groups.

Somewhat unexpectedly, when the fungus was challenged with the unsaturated piperidine derivative, *N*-benzyloxycarbonyl-1,2,3,6-tetrahydropyridine **8**, no biohydroxylation was noted but unreacted starting material was recovered. This result was surprising since both allylic hydroxylation and epoxidations[17] are known to be catalysed by P-450 monoxygenases.

Conclusion

This study has established that the regioselectivity of hydroxylation by *Beauveria bassiana* ATCC 7159, an organism much used for biotransformations of this type, may be significantly altered by changes to the linker attaching the putative hydroxylation site to the aromatic recognition group. It appears that regioselectivity is not only determined by distance of hydroxylation site to the carbonyl group, which is the same for both *N*-benzoyl and *N*-Cbz protected piperidines. The distance to the aromatic sidechain is also important, suggesting that the active site of the enzyme contains a defined aromatic binding pocket.

Experimental

Apparatus and chemicals

All chemicals were purchased from Aldrich Chemical Company, Poole, Dorset UK unless specified otherwise. ^{1}H and ^{13}C NMR analysis was performed on a Bruker AC250 spectrometer (at 250 MHz and 63 MHz respectively) and on a WH-360 spectrometer (at 360 MHz and 90 MHz). Spectra were recorded in deuterochloroform. Chemical shifts are quoted in parts per million (ppm) and these chemical shifts were referenced internally (^{1}H, C*H*Cl$_3$, 7.62 ppm; ^{13}*C*HCl$_3$, 77.0 ppm). Coupling constants (*J*) are quoted in Hz. Carbon multiplicity was established by DEPT (distortionless enhancement by polarisation transfer). Infra-red spectra were recorded on a Perkin-Elmer FT-IR spectrometer (Paragon 1000) as thin films. Mass spectra were run using electron impact (EI) on a Finnigan 4600 instrument for nominal mass and a Kratos MS50TC instrument for high

resolution spectra. Thin layer chromatography was performed on glass sheets coated with silica gel Merck 60F-254 (0.24 mm, Art. 5715). Components were detected by UV (254 nm) and visualised by treating the plate with ammonium molybdate solution and heating. Wet flash chromatography was carried out on silica gel (Merck 9385, Kieselgel 60). Gas chromatography was carried out using a Hewlett-Packard 6890 series GC system (employing a HP-5 5% phenyl methyl siloxane capillary column (30.0 m × 320 μm × 0.25 μm)). Oven temperature gradient from 200 °C (2 min) ramp to 250 °C (20 °C min^{-1}; 10 min); inlet temperature 200 °C and detector temperature 300 °C. The HPLC system used was a Waters 486 controller using Millenium software using Phenomenex ODS-2 5 μ column (25 cm × 4.6 mm) using CH_3CN–0.1% NH_4OAc solution as eluent.

General method for the preparation of Cbz-protected alkylpiperidines

The substituted piperidine (0.010 mol) and benzyl chloroformate (0.012 mol) were added to a stirred solution of potassium carbonate (0.030 mol) in tetrahydrofuran (50 cm^3) under an atmosphere of argon. After stirring for 1 h, water (10 cm^3) was added and the solution was stirred for 1 h. Water (150 cm^3) was then added and the mixture extracted with ethyl acetate (3 × 150 cm^3). The combined organic extracts were washed with saturated sodium carbonate solution (160 cm^3) and brine (2 × 120 cm^3) and dried over anhydrous $MgSO_4$. After filtration, the solvent was removed *in vacuo* and the residue purified by wet flash chromatography using petroleum ether–ethyl acetate mixture as eluent.

***N*-Benzyloxycarbonylpiperidine 1.**[18] Carbamate (**1**) was isolated as a colourless oil (2.04 g, 93%); ν_{max} 1701 cm^{-1}; δ_H 1.46–1.63 (6H, m, C(3)H_2, C(4)H_2 and C(5)H_2), 3.41–3.46 (4H, m, C(2)H_2 and C(6)H_2), 5.12 (2H, s, CH_2Ph), 7.25–7.38 (5H, m, aromatic); δ_C 24.1 (t, C3 and C5), 25.4 (t, C4), 44.6 (t, C2 and C6), 66.6 (t, CH_2Ph), 127.6 (d, aromatic), 127.6 (d, aromatic), 128.2 (d, aromatic), 136.8 (s, aromatic), 155.1 (s, C=O); *m/z* (EI) required 219.12593, found 219.12558, 219 (14%, M^+), 128 (12, M − CH_2Ph), 91 (100, $PhCH_2$), 84 (10, M − CO_2CH_2Ph).

***N*-Benzyloxycarbonyl-4-methylpiperidine 2.** Carbamate (**2**) was isolated as a colourless oil (2.08 g, 89%); ν_{max} 1701 cm^{-1}; δ_H (250 MHz) 0.93 (3H, d, *J* 6.5, CH_3), 1.03–1.18 (1H, m, C(4)H), 1.46–1.61 (4H, m, C(3)H_2 and C(5)H_2), 2.75 (2H, ddd, *J* 2.5, 13 and 13, C(2)Hax and C(6)Hax), 4.13 (2H, br d, *J* 13, C(2)Heq and C(6)Heq), 5.11 (2H, s, CH_2Ph), 7.24–7.36 (5H, m, aromatic); δ_C 63 MHz) 21.7 (q, CH_3), 30.8 (d, C4), 33.8 (t, C3 and C5), 44.1 (t, C2 and C6), 66.8 (d, CH_2Ph), 127.7 (d, aromatic), 127.8 (d, aromatic), 128.3 (d, aromatic), 136.9 (s, aromatic), 155.2 (s, C=O); *m/z* (EI) required 233.14158, found 233.14262, 233 (81%, M^+), 218 (4, M − CH_3), 142 (78, M − CH_2Ph), 126 (70, M − OCH_2Ph), 98 (73, M − CO_2CH_2Ph), 91 (62, CH_2Ph), 77 (13, Ph).

***N*-Benzyloxycarbonyl-3-methylpiperidine 3.** Carbamate (**3**) was isolated as a colourless oil (2.14 g, 92%); ν_{max} 1701 cm^{-1}; δ_H (250 MHz) 0.87 (3H, d, *J* 6.5, CH_3), 1.01–1.13 (1H, m, C(3)H), 1.39–1.81 (4H, m, C(4)H_2 and C(5)H_2), 2.42 (1H, dd, *J* 11 and 13, C(2)Hax), 2.71–2.81 (1H, m, C(6)Hax), 4.00–4.08 (2H, m, C(2)Heq and C(6)Heq), 5.12 (2H, s, CH_2Ph), 7.25–7.37 (5H, m, aromatic); δ_C (63 MHz) 18.7 (q, CH_3), 25.7 (d, C3), 30.8 (t, C4 or C5), 32.7 (t, C4 or C5), 44.2 (t, C2 or C6), 51.1 (t, C2 or C6), 66.7 (t, CH_2Ph), 127.6 (d, aromatic), 127.7 (d, aromatic), 128.3 (d, aromatic), 136.9 (s, aromatic), 155.1 (s, C=O); *m/z* (EI) required 233.14158, found 233.14109, 233 (35%, M^+), 142 (46, M − CH_2Ph), 126 (26, M − OCH_2Ph), 98 (27, M − CO_2CH_2Ph), 91 (100, CH_2Ph).

***N*-Benzyloxycarbonyl-*cis*-2,6-dimethylpiperidine 5.**[19] Carbamate (**5**) was isolated as a colourless oil (2.19 g, 89%); ν_{max} 1693 cm^{-1}; δ_H (250 MHz) 1.20 (6H, d, *J* 7, 2 × CH_3), 1.41–1.77 (6H, m, C(3)H_2, C(4)H_2 and C(5)H_2), 4.32–4.38 (2H, br m, C(2)H and C(6)H), 5.13 (2H, s, CH_2Ph), 7.25–7.36 (5H, m, aromatic); δ_C 13.5 (t, C4), 20.7 (q, C7 and C8), 29.8 (t, C3 and C5), 45.9 (d, C2 and C6), 66.6 (t, CH_2Ph), 127.5 (d, aromatic), 127.6 (d, aromatic), 128.3 (d, aromatic), 137.0 (s, CH_2Ph), 155.5 (s, C=O); *m/z* (EI) required 247.157229, found 247.15832, 247 (4%, M^+), 32 (22, M − CH_3), 156 (13, M − CH_2Ph), 91 (100, $PhCH_2$).

***N*-Benzyloxycarbonyl-2-ethylpiperidine 6.** Carbamate (**6**) was isolated as a colourless oil (2.28 g, 92%); ν_{max} 1693 cm^{-1}; δ_H (250 MHz) 0.83 (3H, t, *J* 7.5, C(8)H_3), 1.34–1.80 (8H, m, C(3)H_2, C(4)H_2, C(5)H_2 and C(7)H_2), 2.75–2.86 (1H, m, C(6)Hax), 4.02–4.07 (1H, br m, C(2)Heq or C(6)Heq), 4.16–4.23 (1H, br m, C(2)Heq or C(6)Heq), 5.17 (2H, s, CH_2Ph), 7.25–7.37 (5H, m, aromatic); δ_C (63 MHz) 10.6 (q, C8), 18.8 (t, C7), 22.4 (t, C3, C4 or C5), 25.5 (t, C3, C4 or C5), 27.9 (t, C3, C4 or C5), 38.9 (t, C6), 52.2 (d, C2), 66.7 (t, CH_2Ph), 127.6 (d, aromatic), 127.6 (d, aromatic), 128.3 (d, aromatic), 137.0 (s, aromatic), 155.6 (s, C=O); *m/z* (EI) required 247.15723, found 247.15706, 247 (6%, M^+), 218 (87%, M − CH_2CH_3), 156 (9, M − CH_2Ph), 140 (5, M − OCH_2Ph), 112 (6, M − CO_2CH_2Ph), 91 (67, CH_2Ph).

***N*-Benzyloxycarbonyl-3,3-dimethylpiperidine 7.** Carbamate (**7**) was isolated as a colourless oil (2.20 g, 89%); ν_{max} 1701 cm^{-1}; δ_H 0.89 (6H, s, C(7)H_3 and C(8)H_3), 1.32–1.37 (2H, m, C(4)H_2 or C(5)H_2), 1.55–1.60 (2H, m, C(4)H_2 or C(5)H_2), 3.13 (2H, s, C(2)H_2), 3.40 (2H, dd, *J* 5.5 and 5.5, C(6)H_2), 5.12 (2H, s, CH_2Ph), 7.25–7.36 (5H, m, aromatic); δ_C 25.8 (q, C7 and C8), 30.8 (s, C3), 37.6 (t, C4), 44.3 (t, C2 or C6), 55.3 (t, C2 or C6), 66.7 (t, CH_2Ph), 127.6 (d, aromatic), 127.7 (d, aromatic), 128.3 (d, aromatic), 137.0 (s, aromatic), 155.5 (s, C=O); *m/z* (EI) required 247.15723, found 247.15813, 247 (36%, M^+), 156 (42, M − CH_2Ph), 140 (20, M − OCH_2Ph), 112 (17, M − CO_2-CH_2Ph), 91 (63, $PhCH_2$).

Maintenance and growth of microorganism

Beauveria bassiana ATCC 7159 was obtained from the American Type Culture Collection, USA. The organism was maintained on malt extract agar plates at room temperature and these were subcultured at regular intervals. A loop of fungus was used to inoculate 60 cm^3 of sterile medium containing 7.5 g dm^{-3} corn steep solids and 10 g dm^{-3} glucose in distilled water adjusted to pH 4.85 in a 250 cm^3 Erlenmeyer flask. After three days growth on an orbital shaker at 200 rpm at 25 °C, a culture was used to inoculate 600 cm^3 of the same medium in a 2 dm^3 Erlenmeyer flask. This culture was again grown for three days.

General biotransformation procedure

A solution of 66 mg substrate in 1 cm^3 ethanol was prepared and this was added to the three day old culture of *Beauveria bassiana* ATCC 7159. After a further three days incubation as above, the fungal cells were removed from the broth by centrifugation and the supernatant extracted into ethyl acetate. Purification of metabolites was routinely carried out using flash silica chromatography with petroleum ether–ethyl acetate gradients as eluent.

Biohydroxylation of *N*-benzyloxycarbonylpiperidine **1** produced hydroxylated product (**9**) which was isolated as a colourless oil (100 mg, 33%). The product **9** was identical to previously reported material as judged by NMR spectroscopy.[20]

Biohydroxylation of *N*-benzyloxycarbonyl-4-methylpiperidine 2. Hydroxylated product (**10**) was isolated as a pale yellow solid (130 mg, 45%); mp 76 °C; δ_H (250 MHz) 1.24 (3H, s, CH_3), 1.45–1.56 (5H, m, C(3)H_2, C(5)H_2 and C(4)OH), 3.34–3.23

(2H, dt, *J* 7 and 13, C(2)Hax and C(6)Hax), 3.80 (2H, br d, *J* 13, C(2)Heq and C(6)Heq), 5.11 (2H, s, CH_2Ph), 7.25–7.36 (5H, m, aromatic); δ_C (63 MHz) 30.0 (q, CH_3), 38.2 (t, C3 and C5), 40.2 (t, C2 and C6), 66.9 (t, CH_2Ph), 67.7 (s, C4), 127.7 (d, aromatic), 127.8 (d, aromatic), 128.3 (d, aromatic), 136.7 (s, aromatic), 155.1 (s, C=O); *m/z* (EI) required 249.13649, found 249.13637, 249 (44%, M^+), 158 (14, M − $PhCH_2$), 142 (16, M − $PhCH_2$), 91 (100, $PhCH_2$).

Biohydroxylation of *N*-benzyloxycarbonyl-3-methylpiperidine 3. Three products **11a–c** were isolated from this biohydroxylation, two of which (**11a** and **11c**) were found to be very closely related and thus difficult to separate. These two products were separated by subjecting the mixture to acetylation conditions (10 mol equiv. acetic anhydride, 5 mol equiv. pyridine and catalytic dimethylaminopyridine) under which conditions one of the products was acetylated **11c** and one was left untouched **11a**. The products were then separated by preparative HPLC.

Product **11a** was isolated as a colourless oil (10 mg, 3%); δ_H (360 MHz) 1.21 (3H, s, CH_3), 1.49–1.79 (5H, m, C(3)OH, C(4)H_2 and C(5)H_2), 2.96–3.03 (2H, br m, C(2)Hax and C(6)Hax), 3.68 (1H, br s, C(2)Heq), 3.84 (1H, ddd, *J* 4.5, 4.5 and 13, C(6)Heq), 5.13 (2H, s, CH_2Ph), 7.28–7.35 (5H, m, aromatic); *m/z* (EI) required 249.13649, found 249.13725, 249 (5%, M^+), 114 (26, M − $PhCH_2OCO$), 91 (100, $PhCH_2$).

Hydroxylated product **11b** was isolated as a colourless oil (10 mg, 3%); δ_H (360 MHz) 0.99 (3H, d, *J* 6.5, CH_3), 1.43–1.59 (3H, br m, three of C(3)H, C(4)OH and C(5)H_2), 1.91 (1H, br dd, *J* 3.5 and 13, C(3)H, C(4)OH or C(5)H), 2.53 (1H, br s, C(2)Hax or C(6)Hax), 2.90 (1H, ddd, *J* 3, 12 and 14, C(4)H), 3.30 (1H, br m, C(2)Hax or C(6)Hax), 4.05–4.13 (2H, br m, C(2)Heq and C(6)Heq), 5.11–5.12 (2H, d, *J* 2, CH_2Ph), 7.28–7.36 (5H, m, aromatic); *m/z* (EI) required 249.13649, found 249.13616, 249 (8%, M^+), 158 (11, M − $PhCH_2$), 142 (M − $PhCH_2O$), 91 ($PhCH_2$).

The *O*-acetylated product of **11c** was isolated as a colourless oil (5 mg); δ_H (360 MHz) 0.88 (3H, d, *J* 12.5, CH_3), 1.67–1.93 (3H, br m, C(3)H and C(5)H_2), 2.07 (3H, s, CH_3CO_2), 3.00–3.15 (1H, br m, C(2)Hax or C(6)Hax), 3.25–3.28 (1H, br m, C(2)Hax or C(6)Hax), 3.70 (1H, br s, C(2)Heq or C(6)Heq), 3.75 (1H, ddd, *J* 4.5, 4.5 and 13.5, C(2)Heq or C(6)Heq), 5.01 (1H, ddd, *J* 3, 3 and 5, C(4)Heq), 5.12 (2H, d, *J* 2, CH_2Ph), 7.28–7.39 (5H, m, aromatic); *m/z* (EI) required 291.14706, found 291.14768; 291 (3%, M^+), 200 (11, M − $PhCH_2$), 91 (100, $PhCH_2$).

Biohydroxylation of *N*-Benzyloxycarbonyl-2-methylpiperidine 4. Hydroxylated product **12a** was isolated as a colourless oil (20 mg, 7%); δ_H (250 MHz) 1.35 (3H, d, *J* 7, CH_3), 1.46–1.91 (5H, m, C(3)H_2, C(4)OH and C(5)H_2), 3.34 (1H, ddd, *J* 5, 12.5 and 13.5, C(6)Hax), 3.90 (1H, ddd, *J* 3.5, 4 and 12.5, C(6)Heq), 4.16 (1H, q, *J* 3, C*H*OH), 4.31–4.42 (1H, m, C(2)Heq), 5.12 (2H, s, CH_2Ph), 7.25–7.36 (5H, m, aromatic); δ_C (90 MHz) 19.1 (q, CH_3), 32.2 (t, C3 or C5), 33.5 (t, C3 or C5), 36.4 (t, C6), 45.7 (d, C2), 64.7 (d, CHOH), 66.8 (t, CH_2Ph), 127.7 (d, aromatic), 127.8 (d, aromatic), 128.4 (d, aromatic), 136.9 (s, aromatic), 155.2 (s, C=O); *m/z* (EI) required 249.13649, found 249.136337, (FAB) 234 (4%, M − CH_3), 158 (15, M − $PhCH_2$), 142 (31, M − $PhCH_2O$), 114 (2, M − $PhCH_2CO_2$), 91 (100, $PhCH_2$).

Hydroxylated product **12b** was isolated as a colourless oil (20 mg, 7%); δ_H (360 MHz) 1.12 (3H, d, *J* 7, CH_3), 1.43–1.78 (5H, m, C(3)OH, C(4)H_2 and C(5)H_2), 2.79 (1H, ddd, *J* 3, 13 and 13.5, C(6)Hax), 3.74–3.80 (1H, m, C*H*OH), 3.95 (1H, br d, *J* 12.5, C(6)Heq), 4.51 (1H, q, *J* 6.5, C(2)H), 5.12 (2H, s, CH_2Ph), 7.25–7.38 (5H, m, aromatic); δ_C (90 MHz) 9.2 (q, CH_3), 24.0 (t, C5 or C4), 27.1 (t, C5 or C4), 37.6 (t, C6), 51.0 (d, C2), 67.0 (t, CH_2Ph), 69.0 (d, CHOH), 127.7 (d, aromatic), 127.9 (d, aromatic), 128.4 (d, aromatic), 136.7 (s, aromatic), 155.3 (s, C=O); *m/z* (EI) required 249.13649, found 249.13637, (APCI†) 250 (100%, M + 1), 217 (3, M − $OHCH_3$), 158 (7, M − $PhCH_2$).

Biohydroxylation of *N*-benzyloxycarbonyl-*cis*-2,6-dimethylpiperidine 5. Hydroxylated product **13** was isolated as a colourless oil (10 mg, 5%); δ_H (360 MHz) 1.25 (6H, d, *J* 7, 2 × CH_3), 1.42–1.60 and 1.86–1.90 (5H, m, C(3)H_2, C(4)OH and C(5)H_2), 4.20 (1H, tt, *J* 11 and 4, C*H*OH), 4.49–4.57 (2H, m, C(2)Heq and C(6)Heq), 5.14 (2H, s, CH_2Ph), 7.23–7.38 (5H, m, aromatic); δ_C (63 MHz, DEPT) 21.7 (q, 2 × CH_3), 39.4 (t, C3 and C5), 47.2 (d, C2 and C6), 61.4 (d, CHOH), 66.9 (t, CH_2Ph), 127.7 (d, aromatic), 127.8 (d, aromatic), 128.4 (d, aromatic); *m/z* (EI) required 263.15214, found 263.15203, (FAB) 264 (M + 1).

Biohydroxylation of *N*-benzyloxycarbonyl-2-ethylpiperidine 6. Hydroxylated product **14** was isolated as a colourless oil (130 mg, 45%); δ_H (360 MHz) 0.85 (3H, t, *J* 7.5, C(8)H_3), 1.23–1.66 (5H, m, C(3)H, C(4)OH, C(5)H and C(7)H_2), 1.92 (2H, dd, *J* 2.5 and 12.5, C(3)H and C(5), 2.85 (1H, ddd, *J* 2.5, 12.5 and 12.5, C(6)Hax), 3.91 (1H, tt, *J* 4.5 and 11.5, C*H*OH), 4.16 (1H, br d, *J* 12.5, C(6)Heq), 4.33 (1H, br m, C(2)Heq), 5.12 (2H, d, *J* 2, CH_2Ph), 7.25–7.37 (5H, m, aromatic); δ_C (90 MHz) 10.7 (q, C8), 23.8 (t, C7), 35.0 (C3 and C5), 37.6 (t, C6), 52.8 (d, C2), 65.0 (t, CHOH), 67.0 (t, CH_2Ph), 127.6 (d, aromatic), 127.8 (d, aromatic), 128.3 (d, aromatic), 136.8 (s, aromatic), 155.4 (s, C=O); *m/z* (EI) required 263.15214, found 263.15253, (APCi) 264 (12%, M + 1), 220 (M − CH_3CH_2), 172 (7, M − $PhCH_2$), 156 (23, M − $PhCH_2O$), 91 (100, $PhCH_2$).

Biohydroxylation of *N*-benzyloxycarbonyl-3,3-dimethylpiperidine 7. Hydroxylated product **15** was isolated as a colourless oil (134 mg, 48%); δ_H (250 MHz; 345 K) 0.89 (3H, s, C(7)H_3 or C(8)H_3), 0.95 (3H, s, C(7)H_3 or C(8)H_3), 1.51–1.64 (2H, m, C(5)H and C(4)OH), 1.71–1.82 (1H, m, C(5)H), 2.85 (1H, d, *J* 13.5, C(2)Hax), 3.12–3.23 (1H, m, C(6)Hax), 3.40 (1H, dd, *J* 8.5 and 4.0, C*H*OH), 3.56 (1H, dd, *J* 1.5 and 13.5, C(2)Heq), 3.81–3.91 (1H, m, C(6)Heq), 5.13 (2H, s, CH_2Ph), 7.24–7.36 (5H, m, aromatic); δ_H (63 MHz) 18.4 (q, C7 or C8), 24.2 (q, C7 or C8), 29.5 (s, C3), 35.8 (t, C5), 41.5 (t, C2 or C6), 52.6 (t, C2 or C6), 66.9 (t, CH_2Ph), 74.7 (d, CHOH), 127.7 (d, aromatic), 127.8 (d, aromatic), 128.3 (d, aromatic), 136.7 (s, aromatic), 155.4 (s, C=O); *m/z* required 263.15214, found 263.15254, 263 (10%, M^+), 172 (5, M − $PhCH_2$), 128 (3, CO_2CH_2Ph), 91 (100, CH_2Ph), 77 (4, Ph).

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† APCI = Atmospheric pressure chemical ionisation.

References

1 E. W. Weiler, M. Droste, J. Eberle, H. J. Halfmann and A. Weber, *Appl. Microbiol. Biotechnol.*, 1987, **27**, 252.
2 R. A. Johnson, M. E. Herr, H. C. Murray, L. M. Reineke and G. S. Fonken, *J. Org. Chem.*, 1968, **33**, 3195; R. Furstoss, A. Archelas and B. Waegell, *Tetrahedron Lett.*, 1981, **22**, 445; W. Carruthers, J. D. Prail, S. M. Roberts and A. J. Willetts, *J. Chem. Soc., Perkin Trans. 1*, 1990, 2854; C. F. Palmer, B. Webb, S. Broad, S. Casson, R. McCague, A. J. Willetts and S. M. Roberts, *Bioorg. Med. Chem. Lett.*, 1997, **7**, 1299.
3 G. S. Fonken and R. A. Johnson, in *Chemical Oxidations with Microorganisms*, VCH, Germany, 1972.
4 R. A. Johnson, H. C. Murray, L. M. Reineke and G. S. Fonken, *J. Org. Chem.*, 1969, **34**, 2279.
5 K. Tadano, Y. Iimura and T. Suami, *J. Carbohydr. Chem.*, 1985, **4**, 129.

6 B. Ringdahl, U. F. W. Ohnsorge and J. C. Craig, *J. Chem. Soc., Perkin Trans. 2*, 1981, 697.
7 A. de Raat, H. Griengl, M. Petsch, P. Plachota, N. Schoo, H. Weber, G. Braunegg, I. Kopper, M. Kreiner, A. Zeiser and K. Kieslich, *Tetrahedron: Asymmetry*, 1996, **7**, 467; A. de Raat, H. Griengl, M. Petsch, P. Plachota, N. Schoo, H. Weber, G. Braunegg, I. Kopper, M. Kreiner and A. Zeiser, *Tetrahedron: Asymmetry*, 1996, **7**, 473; A. de Raat, H. Griengl, M. Petsch, P. Plachota, N. Schoo, H. Weber, G. Braunegg, I. Kopper, M. Kreiner and A. Zeiser, *Tetrahedron: Asymmetry*, 1996, **7**, 491.
8 D. D. Keith, J. A. Tortora, K. Ineichen and W. Leimgruber, *Tetrahedron*, 1975, **31**, 2633; D. A. Evans and J. A. Ellman, *J. Am. Chem. Soc.*, 1989, **111**, 1063; K. E. Harding, T. H. Marman and D.-H. Nam, *Tetrahedron Lett.*, 1988, **29**, 1627.
9 N. Floyd, F. Munyemana, S. M. Roberts and A. J. Willetts, *J. Chem. Soc., Perkin Trans. 1*, 1993, 881.
10 J. D. Fourneron, A. Archelas and R. Furstoss, *J. Org. Chem.*, 1989, **54**, 2478.
11 R. A. Johnson, *J. Org. Chem.*, 1968, **33**, 3627.
12 Y. L. Chow, C. J. Colon and J. N. S. Tam, *Can. J. Chem.*, 1968, **46**, 2821.
13 V. Gotor, M. Quiros, L. Ramon, J. Frigola and R. Fernandez, *Tetrahedron*, 1997, **53**, 6421.
14 H. L. Holland, M. Conn, P. Chinna Chenchaiah and F. M. Brown, *Tetrahedron Lett.*, 1988, **29**, 6393.
15 B. Vigne, A. Archelas and R. Furstoss, *Tetrahedron*, 1991, **47**, 1447.
16 R. Furstoss, A. Archelas, J. D. Fourneron and B. Vigne, in *Enzymes as Catalysts in Organic Chemistry*, NATO ASI Series, ed. P. Schneider, 1986, **178**, 361.
17 R. A. Johnson, M. E. Herr, H. C. Murray, C. G. Chidester and F. Han, *J. Org. Chem.*, 1992, **57**, 7209.
18 A. C. Pierce and M. M. Joullie, *J. Org. Chem.*, 1963, **28**, 658; G. Barcelo, J.-P. Senet, G. Sennyey, J. Bensoam and A. Loffet, *Synthesis*, 1986, 627.
19 K. E. Harding and T. H. Marman, *J. Org. Chem.*, 1984, **49**, 2838.
20 C. S. Cooper, P. L. Klock, D. T. W. Chu, D. J. Hardy and R. N. Swanson, *J. Med. Chem.*, 1992, **35**, 1392; L. Alig, A. Edenhofer, P. Hadváry, M. Hürzeler, D. Knopp, M. Müller, B. Steiner, A. Trzeciak and T. Weller, *J. Med. Chem.*, 1992, **35**, 4393.
21 A. M. Morello and A. D. Ward, *Aust. J. Chem.*, 1995, **48**, 445.
22 S. V. D'Andrea, E. T. Michalson, J. P. Freeman, C. G. Chidester and J. Szmuszkovicz, *J. Org. Chem.*, 1991, **56**, 3133.

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Biohydroxylation Reactions Catalyzed by Enzymes and Whole-Cell Systems

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The biohydroxylation of a number of cyclic substrates (**3–24**) containing aromatic side chains was used to compare substrate specificity and selectivity of hydroxylation using microbial enzymes and whole-cell biocatalysts. In general, the regioselectivity of reaction was remarkably similar between the different catalysts in that little aromatic or benzylic, but significant aliphatic hydroxylation was observed. However, a more detailed investigation of isolated products showed complementary substrate specificity, functional group compatibility, and regioselectivity of hydroxylation. Substrate specificity and regioselectivity could be further modulated by small changes to the nature of the aromatic side chain, which appears to play an important role in substrate recognition.

The hydroxylation of nonfunctionalized hydrocarbon centers using biological systems is recognized as an important biotransformation (*1*). This reaction has traditionally been achieved using whole-cell systems, but as more isolated enzyme systems and their three-dimensional structure are now becoming available, enzymes can be used for catalysis and are particularly important for studying substrate recognition and regio- and stereoselectivity of hydroxylation. The regioselectivity of hydroxylation is of particular importance to these biotransformations, because substrates often have a number of potential hydroxylation sites and thus a very high degree of selectivity is required. In our studies, we have looked at a particular class of cyclic substrates bearing aromatic side chains (**3–24**) and compared the selectivity of hydroxylation in defined enzymatic systems with that of whole-cell systems.

BIOHYDROXYLATIONS USING ISOLATED ENZYMES

The best studied of the cell-free hydroxylase systems is the cytochrome P450cam monooxygenase from *Pseudomonas putida* which catalyzes the hydroxylation of camphor **1** to 5-*exo*-hydroxy camphor **2** (Scheme 1). This enzyme was first reported in the 1960s by Gunsalus and colleagues and has been widely used as a model system because it is easily obtained as a soluble protein from heterologous expression (*2*)

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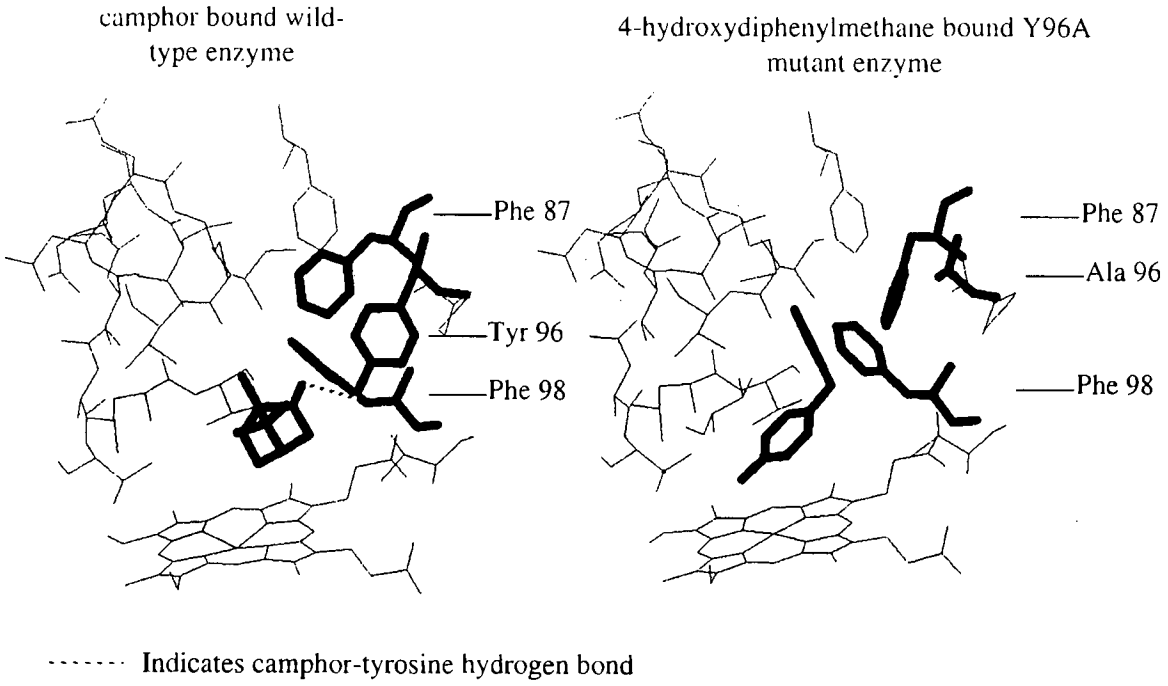
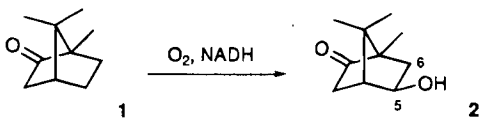


FIG. 1. Active-site residues of cytochrome P450cam monooxygenase. (Left) Wild-type enzyme with camphor bound to the active site (*3*); (right) mutant Y96A with 4-hydroxydiphenyl methane bound to the active site (*14*).

and a high-resolution crystal structure is available (*3*). The reaction is catalyzed with remarkable regio-and stereoselectivity in that only 1 of 10 possible hydroxylation products is formed. This selectivity is due to a combination of the shape of the active site which provides multiple van-der-Waals interactions between enzyme and substrate and a single hydrogen bond between the camphor carbonyl group and tyrosine 96 of the enzyme. The importance of the hydrogen bond has been established by mutagenesis of tyrosine 96 and studies with substrate analogues lacking the carbonyl group (*4*).

In the context of applications to biocatalysis, P450cam suffers from very narrow substrate specificity. It therefore provides an ideal target for redesign by site-directed mutagenesis. Our goal was to broaden the substrate specificity but at the same time retain regioselectivity of hydroxylation. We were particularly interested in aromatic substrates, such as polyaromatic compounds and substrates with aromatic side chains, because of potential applications in bioremediation (*5*) and in the synthesis of chiral intermediates (*6–10*), respectively.

Investigation of the active site of P450cam (Fig. 1, left) suggests that removal of tyrosine 96 would both enlarge the substrate pocket and perhaps even provide an aromatic binding site, since tyrosine 96 in the wild-type structure is flanked by two phenylalanine residues (Phe87 and Phe98, Figure 1), which might provide aromatic



SCHEME 1. Hydroxylation of camphor using P450cam monooxygenase from *Pseudomonas putida.*

stacking interactions. With this in mind, a number of single-site mutants at position 96 were generated, of which the alanine mutant Y96A was the most interesting and hence was studied in most detail (*11,12*).

First, simple aromatic compounds such as naphthalene and diphenylmethane (**3**) were tested as substrates. The hydroxylation of naphthalene was much more efficient with the Y96A mutant than with wild-type enzyme (*13*), and diphenylmethane **3** was almost exclusively converted to 4-hydroxy diphenylmethane (Table 1) by Y96A, whereas no hydroxylation was observed with wild-type enzyme (*11*). No benzylic hydroxylation was observed, which emphasizes that the mode of binding of the substrate to the enzyme active site can be more important for selectivity than C–H bond strength.

Thus, the type of substrate binding we observed with the Y96A mutant (Table 1) suggested to us that removal of the tyrosine aromatic side chain had indeed created a binding pocket for aromatic rings as predicted, where the substrate now takes the place of the former protein tyrosine side chain. To verify this, we conducted crystallographic studies on the Y96A mutant. We have recently obtained the structure of the mutant Y96A with 4-hydroxy diphenylmethane bound to the active site to 1.9 A resolution in collaboration with Malcolm Walkinshaw at Edinburgh University (*14*). These preliminary studies have revealed that, perhaps not surprisingly, some significant rearrangement of active-site residues occurs. Comparison of the wild-type with the Y96A mutant shows that most active-site residues remain in the same place, but very importantly that the phenylalanine 87 side chain has moved into the original site of tyrosine 96 (Fig. 1, right). Thus, the mutation has increased the size of the substrate binding pocket which now appears to be lined at the bottom, away from the active site, with aromatic side chains (Phe 87 and 98). Thus, rather than, as predicted, providing an aromatic pocket at the previous site of tyrosine 96, Phe 98 appears to be stacking against aromatic side chains of the substrate, resulting in regioselectivity of hydroxylation. We are currently investigating cocrystals of Y96A with other substrates to see if this change of active residue orientation is generally observed.

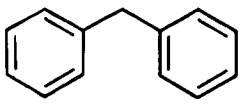
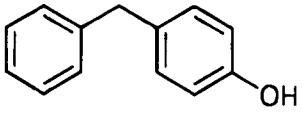
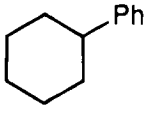
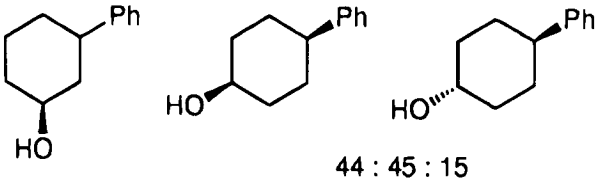
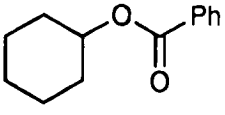
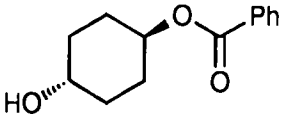
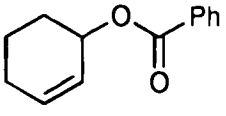
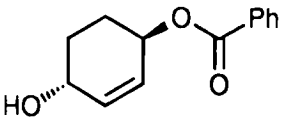
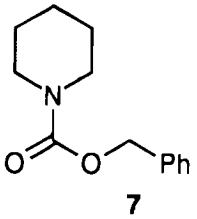
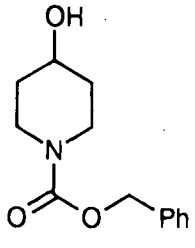
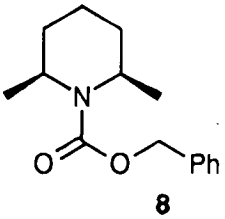
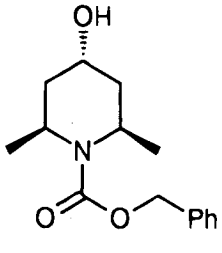
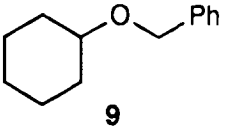
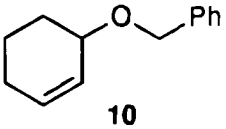
Further evidence for this aromatic stacking interaction comes from studies of the mutant Y96A with substrates **4–9** (Table 1), where no aromatic hydroxylation was observed, but good regioselectivity of hydroxylation of the aliphatic part of the molecule occurred. However, this can also be explained by favorable van-der-Waals interactions of the aliphatic part of the substrate with aliphatic protein side chains (Val 244, Leu 247, Val 295) closer to the active site.

In general, substrates **3–9** in Table 1 demonstrate that the substrate range for hydroxylation has been greatly expanded by the single mutation of tyrosine 96, in particular since none of these compounds bind to wild-type enzyme. The aromatic side chain that is necessary for good binding to the enzyme does not need to be part of the desired substrate structure, but can be a protecting group as in **5–9**, which can be cleaved off after hydroxylation. This allows for the enzyme to catalyze the hydroxylation of functionalized cyclohexane (**5,6,9**) and piperidine (**7,8**) derivatives, which themselves are not substrates.

Another advantage for using aromatic protecting groups in these biotransformations is that they can be used as a further tool to manipulate regioselectivity of hydroxylation.

TABLE 1

Hydroxylation Products Isolated from Incubation of Substrates with the Y96A Mutant of P450cam Monooxygenase from *Pseudomonas putida*

SUBSTRATE	MAJOR PRODUCT(S)
 **3**	
 **4**	 44 : 45 : 15
 **5**	
 **6**	
 **7**	
 **8**	
 **9**	Mixture of products identified by gas chromatography, but not isolated
 **10**	No products observed by gas chromatography

Note. The relative configuration of the stereocenters in products is as indicated. However, absolute configurations have not been determined.

For example, the benzoyl ester of cyclohexanol **5** is selectively oxidized to the 4-hydroxyl derivative, whereas the benzyl ether **9** leads to a mixture of products, or the benzoyl protected cyclohexenol **6** gives the allylic hydroxylation product, whereas incubation of the benzyl ether **10** with Y96A resulted in recovered starting material. It appears that a certain distance between the aromatic side chain and the hydroxylation site is important in these substrates to ensure good selectivity of hydroxylation as comparison of **4** and **5** shows, where **4** gives 44% of 3-hydroxylated product and **5** gives almost exclusively 4-hydroxylated material.

A great practical problem with using isolated enzyme systems for biotransformations is the requirement for coenzymes and cofactors. For example, in the case of cytochrome P450cam monooxygenase discussed here, it is necessary to add the corresponding ferredoxin, ferredoxin reductase, and NADH as a source of electrons. These reactions are therefore only being conducted on a milligram scale and it would be expensive to scale them up much further. In the future, it may be possible to bypass electron transport proteins by driving the reaction electrochemically (*15*), but in the meantime the use of whole-cell systems, whether recombinant (*16*) or in the natural microorganism, is much more practical for preparative-scale biohydroxylations. A few whole-cell systems which we have investigated in our group are discussed below.

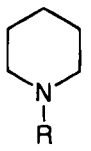
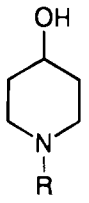
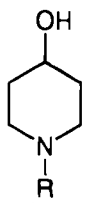
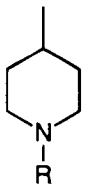
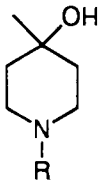
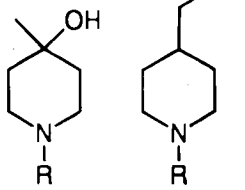
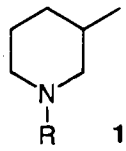
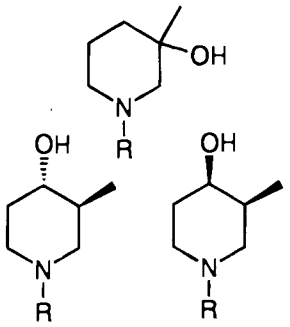
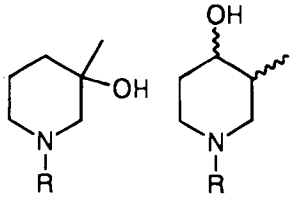
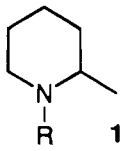
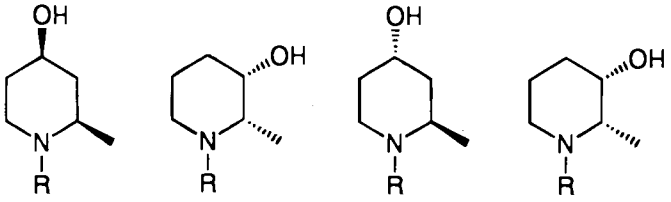
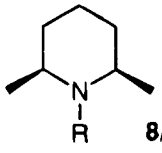
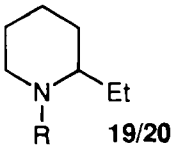
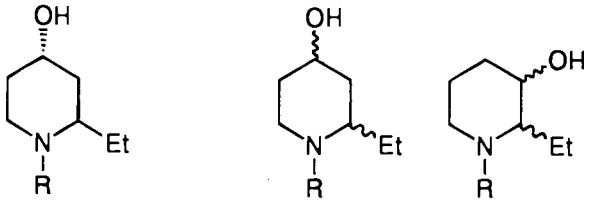
BIOHYDROXYLATIONS USING *B. bassiana*

The entomopathogen *Beauveria bassiana* ATCC 7159 (redesignated from *Sporotrichum sulfurescens*) has been used for a number of biotransformations. Of particular interest to us were studies by Fonken and Johnson at Upjohn Company (*6,10*) on the selective hydroxylation of *N*-benzoyl alkylpiperidines to give some optically active hydroxy alkylpiperidines. This work led to the proposal of a stereochemical model for hydroxylation of these amides, which placed particular importance on the distance between the amide oxygen and the hydroxylation site (*1*). Interestingly, our studies with the isolated enzyme, the cytochrome P450cam mutant Y96A, had given similar products of hydroxylation of the Cbz protected piperidine derivatives **7** and **8** to those observed by Johnson, but modeling studies of the substrates at the active site of the mutant using the available structural data (*17*) would suggest that no protein side chain is suitably placed to form a hydrogen bond with the carbonyl oxygen of the Cbz group. Rather, the size and flexibility of the aromatic substrate protecting group appear to determine regioselectivity of hydroxylation. The comparison of *N*-carboxybenzoyl protected piperidine derivatives with benzoyl protected piperidines using the *B. bassiana* whole-cell hydroxylation system would therefore give us more information on the importance of the aromatic side chain, since the carbonyl group in both substrate classes is placed at the same distance to the different potential hydroxylation sites of the ring. In addition, we were interested to see if the Cbz group was more compatible with whole-cell biotransformations, since it is a much more convenient protecting group for amines than the *N*-benzoyl group.

A comparison of the hydroxylated products from both series of substrates (Cbz and benzoyl protected) is shown in Table 2 (*17*). The protected parent piperidine derivatives **7** and **11** gave both 4-hydroxylated products, but with several of the alkyl substituted piperidines a significant change in the regioselectivity of hydroxylation was observed. Thus, while biohydroxylation of *N*-benzoyl 4-methyl piperidine **13**

TABLE 2

Hydroxylation Products Isolated from Incubation of Alkylpiperidines with *Beauveria bassiana* ATCC 7159

SUBSTRATES	MAJOR PRODUCT(S)	
R = $PhCH_2OCO$-/PhCO-	R = $PhCH_2OCO$-	R = PhCO- [9]
 7/11		
 12/13		
 (+/-) 14/15		
 (+/-) 16/17		
 8/18		
 19/20		

resulted in a 2:1 mixture of the 4-hydroxylmethyl- and the 4-hydroxy-4-methyl-piperidine derivatives, the Cbz compound **12** gave only the 4-hydroxy-4-methyl-piperidine in 48% yield. The 3-methyl derivatives **14** and **15** and the 2-methyl derivatives **16** and **17**, on the other hand, resulted in the same product ratios. The most significant change of regioselectivity was observed with the dimethyl-piperidines **8** and **18** and ethyl-piperidines **19** and **20**. The Cbz derivatives **8** and **19** led exclusively to the 4-hydroxylated materials, whereas the benzoyl derivative **18** gave only 3-hydroxylated product and **20** gave a mixture of isomers.

One concern with whole-cell biotransformations is the lability of certain functional groups to other enzymes in the microorganism. Esters, for example, are likely to be hydrolyzed by microbial enzymes and compounds such as **5** and **6** would therefore not be suitable for whole-cell biotransformations. The Cbz group is potentially labile either to microbial carbamate hydrolases (*18*) or to benzylic hydroxylation (*19*). The results presented in Table 2 suggest that degradation of the Cbz group is generally low. However, we found one exception in substrate **8** where very little hydroxylated product (5%) was isolated together with a large amount (75%) of benzoic acid (*19*), indicating benzylic hydroxylation.

BIOHYDROXYLATIONS USING *Rhodoccocus* spp.

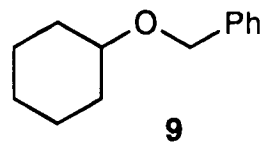
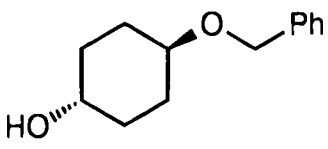
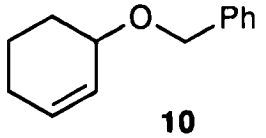
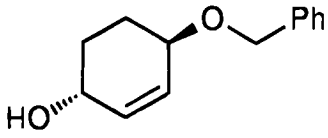
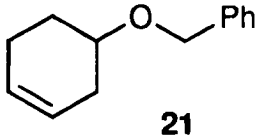
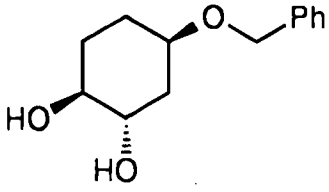
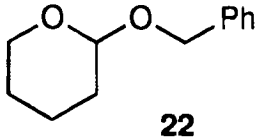
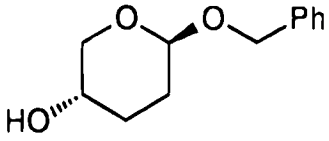
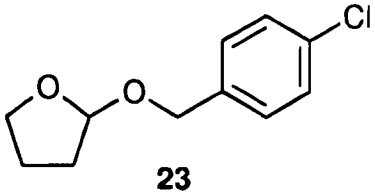
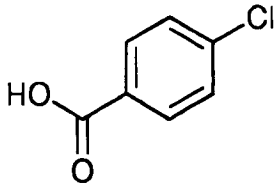
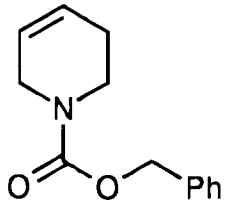
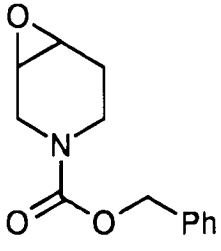
The genus *Rhodococcus* is a group of gram-positive bacteria that is widely distributed in both soil and aquatic environments and is known for their broad metabolic diversity which includes hydrocarbons, many aromatic compounds, and larger molecules such as steroids. A number of *Rhodococcus* strains have been investigated for their ability to catalyze hydroxylation (*20,21*) and have demonstrated that this genus of bacteria has great potential to provide useful hydroxylases. After investigating a number of strains, we decided to study *Rhodococcus rhodochrous* ATCC 19067 and *Rhodococcus* sp NCIMB 9784 in the context of our studies described above.

R. rhodochrous ATCC 19067 had been reported by Cardini and Jurtshuk (*22,23*) to contain an inducible P450 monooxygenase (P450oct) which performed NADH-dependent oxidation of *n*-octane to 1-octanol. We have found that this whole-cell system can also accept a wide range of cyclic substrates such as illustrated in Table 3. Interestingly, the substrates were again exclusively hydroxylated in the aliphatic rather than the aromatic ring. Cbz protected piperidine **7** was hydroxylated at the 4-position as observed by *B. bassiana* and the P450cam mutant Y96A hence suggesting similarities in substrate recognition. However, the *Rhodococcus* showed complementary catalytic activity to the P450cam and the *B. bassiana* systems with a number of substrates: benzylether **9** led to a mixtures of products with the Y96A system, yet gave only a single product with *R. rhodochrous*. Compounds **10**, **21**, and **22** were hydroxylated in good yield by *R. rhodochrous*, yet were not substrates for the Y96A mutant. Cbz protected dehydropiperidine **24** was not a *B. bassiana* substrate but could be oxidized by *R. rhodochrous*. In only one of the cases studied (compound **23**) did we find benzylic hydroxylation.

The other strain we studied, *Rhodococcus* sp NCIMB 9784, contains a hydroxylase which has been reported to generate 6-*endo*-hydroxycamphor from camphor (*24*), although evidence for this reaction has been indirect because of further metabolism of the alcohol product. Because of our interest in the substrate specificity of the

TABLE 3

Hydroxylation Products Isolated from Incubation of Substrates with *Rhodococcus rhodochrous* 9703

SUBSTRATE	MAJOR PRODUCT
 **9**	
 **10**	
 **21**	
 **22**	
 **23**	
 **24**	

Note. The relative configuration of the stereocenters in products is as indicated. However, absolute configurations have not been determined.

cytochrome P450cam hydroxylase from *P. putida*, we have studied this enzymatic activity in more detail. The hydroxylase appears to be remarkably similar in its narrow substrate specificity to the wild-type *P. putida* enzyme in that they are limited to molecules of similar size to camphor and terpenoid substrates. Larger compounds such as the Cbz piperidine 7 are not hydroxylated.

This hydroxylase will therefore not so much be of interest as a new complementary hydroxylation system, but rather be useful for comparison to the P450cam from *P. putida* in order to allow us to further understand factors underlying regioselectivity of hydroxylation. Thus, we have been able to purify a cytochrome P450 monooxygenase from *Rhodococcus* species NCIMB 9784 which accepts camphor as a substrate. The first 16 N-terminal amino acids have been determined by sequencing and little homology was found in this short sequence to the cytochrome P450cam monooxygenase of *P. putida.* Studies toward the cloning and further characterization of this enzyme are currently in progress in our laboratories.

CONCLUSIONS

Comparison of the substrate specificity of the different monooxygenases from microbial sources studied here suggests some surprising similarities in selectivity of hydroxylation, in that the 3- and 4-positions of the aliphatic rings are the most likely to be hydroxylated. However, the substrate selectivity and the regioselectivity of hydroxylation can be fine-tuned either by change of substrate structure (e.g., using the Cbz instead of benzoyl-derivate) or by change of biocatalyst. With cell-free and purified enzyme systems becoming more and more available, we can start to rationalize regioselectivity of hydroxylation and redesign the active site of hydroxylases to suit the particular requirements of reaction selectivity.

ACKNOWLEDGMENTS

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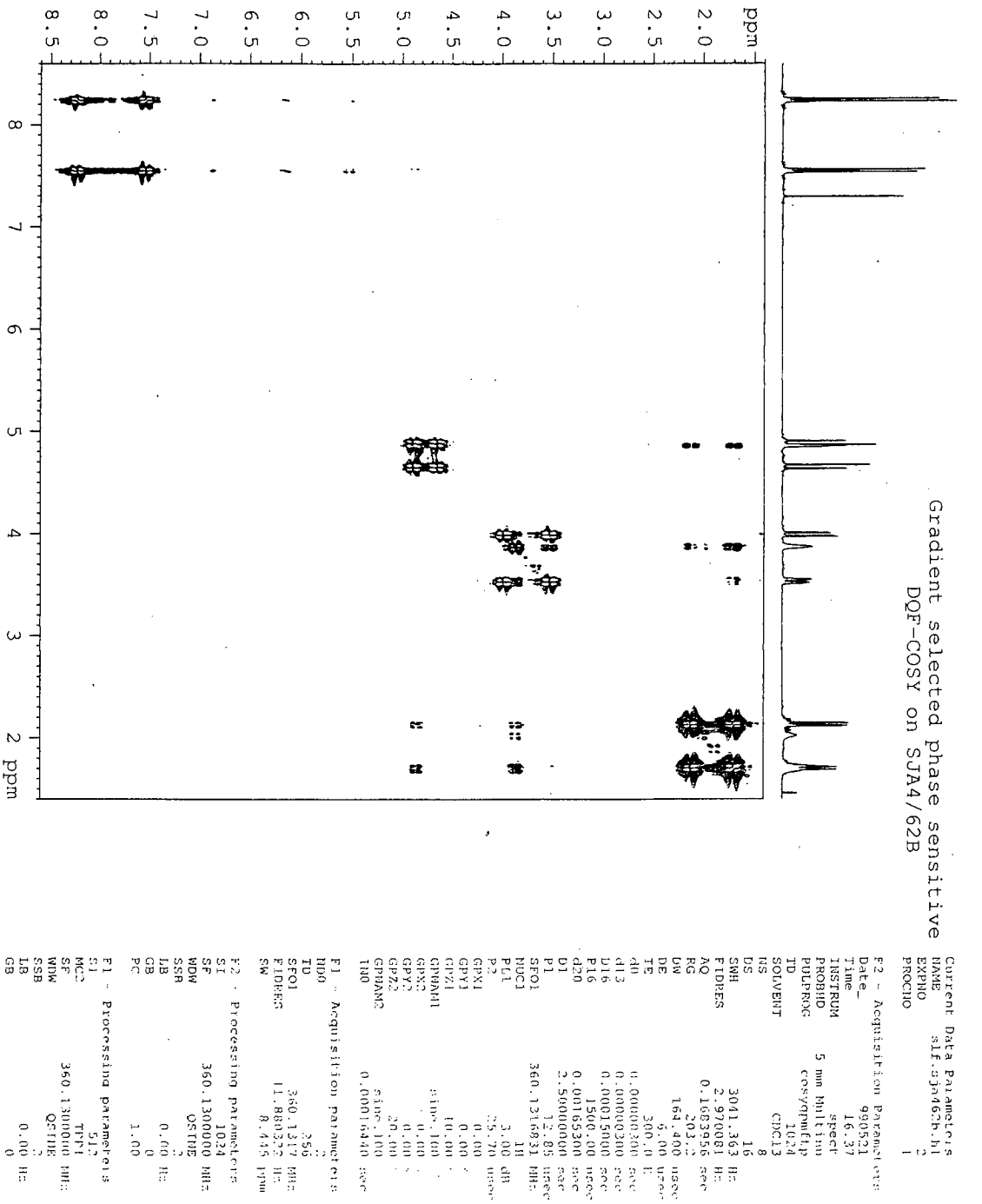
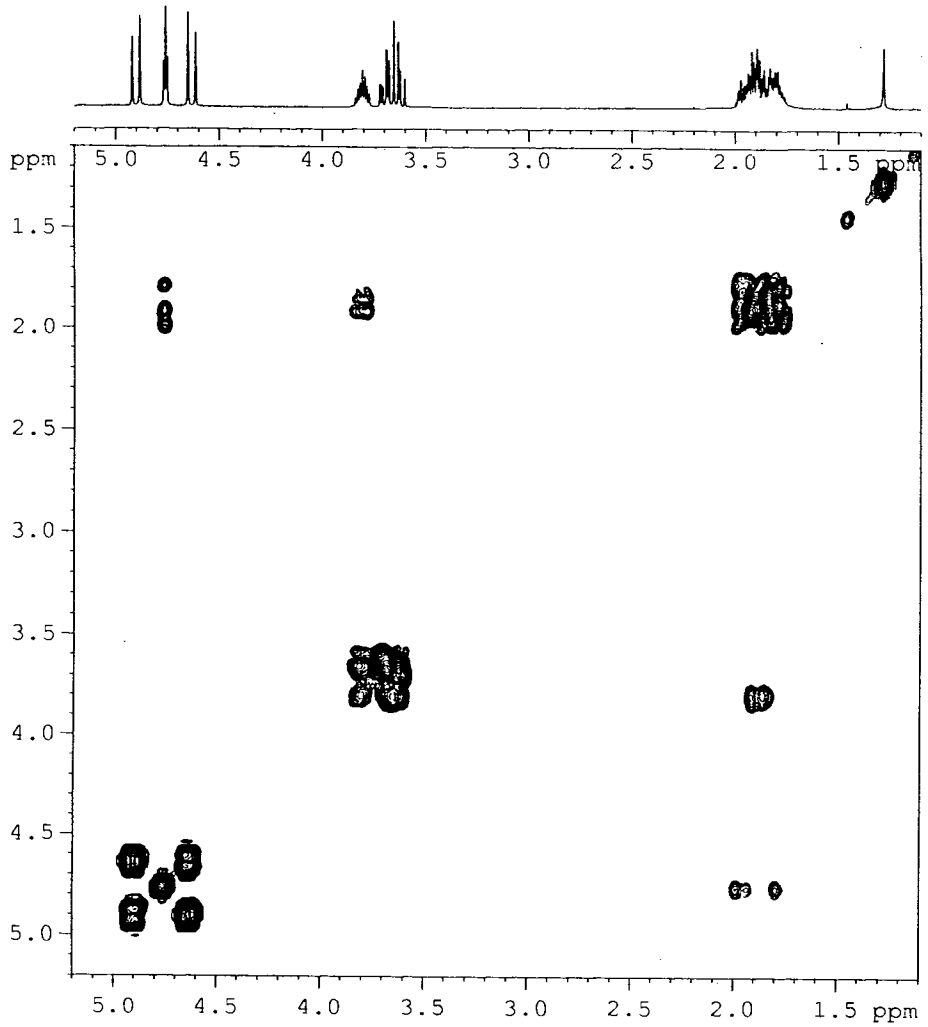
REFERENCES

1. Holland, H. L. (1998) *in* Biotransformations (D. R. Kelly, Ed.), pp. 475–533, Wiley–VCH, Weinheim.
2. Koga, H., Rauchfuss, B., and Gunsalus, I. C. (1985) *Biochem. Biophys. Res. Commun.* **130,** 412–417.
3. Poulos, T. L., Finzel, B. C., and Howard, A. J. (1987) *J. Mol. Biol.* **195,** 697–700.
4. Atkins, W. M., and Sligar, S. G. (1988) *J. Biol. Chem.* **263,** 18842–18849.
5. Kulisch, G. P., and Wilker, V. L. (1991). *Biotechnol. Prog.* **7,** 93–98.
6. Johnson, R. A., Herr, M. E., Murray, H. C., Reineke, L. M., and Fonken, G. S. (1968) *J. Org. Chem.* **33,** 3195.
7. Furstoss, R., Archelas, R., and Waegell, B. (1981) *Tetrahedron Lett.* **22,** 445.
8. Carruthers, W., Prail, J. D., Roberts, S. M., and Willetts, A. J. (1990) *J. Chem. Soc. Perkin Trans. I,* 2854.
9. Palmer, C. F., Webb, B., Broad, S., Casson, S., McCague, R., Willetts, A. J., and Roberts, S. M. (1997) *Bioorg. Med. Chem. Lett.* **7,** 1299.
10. Johnson, R. A., Murray, H. C., Reineke, L. M., and Fonken, G. S. (1969) *J. Org. Chem.* **34,** 2279.
11. Fowler, S., England, P. A., Westlake, A. C. G., Rouch, D. R., Nickerson, D. P., Blunt, C., Braybrook, D., West, S., Wong, L.-L., and Flitsch, S. L. (1994) *J. Chem. Soc. Chem. Commun.,* 2761–2762.
12. Wong, L.-L. (1998) *Curr. Opin. Chem. Biol.* **2,** 263–268.
13. England, P. A., Harford-Cross, C. F., Stevenson, J.-A., Rouch, D., and Wong, L.-L. (1998) *FEBS Lett.* **424,** 271–274.
14. Staines, A., Taylor, P., Flitsch, S. L., and Walkinshaw, M. Unpublished results.
15. Estabrook, R. W., Faulkner, K. M., Shet, M. S., and Fisher, C. W. (1996) *Methods Enzymol.* **272,** 44–51.
16. Sibbesen, O., De Voss, J. J., and Ortiz de Montellano, P. R. (1996) *J. Biol. Chem.* **271,** 22462–22469.

17. Aitken, S. J., Grogan, G., Chow, C. S.-Y., Turner, N. J., and Flitsch, S. L. (1998) *J. Chem. Soc. Perkin Trans. 1* **20,** 3365–3370.
18. Gotor, V., Quiros, M., Liz, R., Frifola, J., and Fernandez, R. (1997) *Tetrahedron* **53,** 6421–6432.
19. Holland, H. L., Conn, M., Chinna Chenchaiah, P., and Brown, F. M. (1988) *Tetrahedron Lett.* **29,** 6393.
20. Banci, L., Bertini, I., Eltis, L. D., and Pierattelli, R. (1993) *Biophys. J.* **65,** 806–813.
21. Nagy, I., Compernolle, F., Ghys, K., Vanderleyden, J., and De Mot, R. (1995) *Appl. Environ. Microbiol.,* 2056–2060.
22. Cardini, G., and Jurtshuk, P. (1968) *J. Biol. Chem. 243,* 6070–6072.
23. Cardini, G., and Jurtshuk, P. (1970) *J. Biol. Chem. 245,* 2789–2796.
24. Chapman, P. J., Meerman, G., Gunsalus, I. C., Srinivasan, R., and Rinehart, K. L. (1966) *J. Am. Chem. Soc.* **88,** 618–619.



^{1}H-^{1}H COSY of alcohol **120**

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PROCNO                 1

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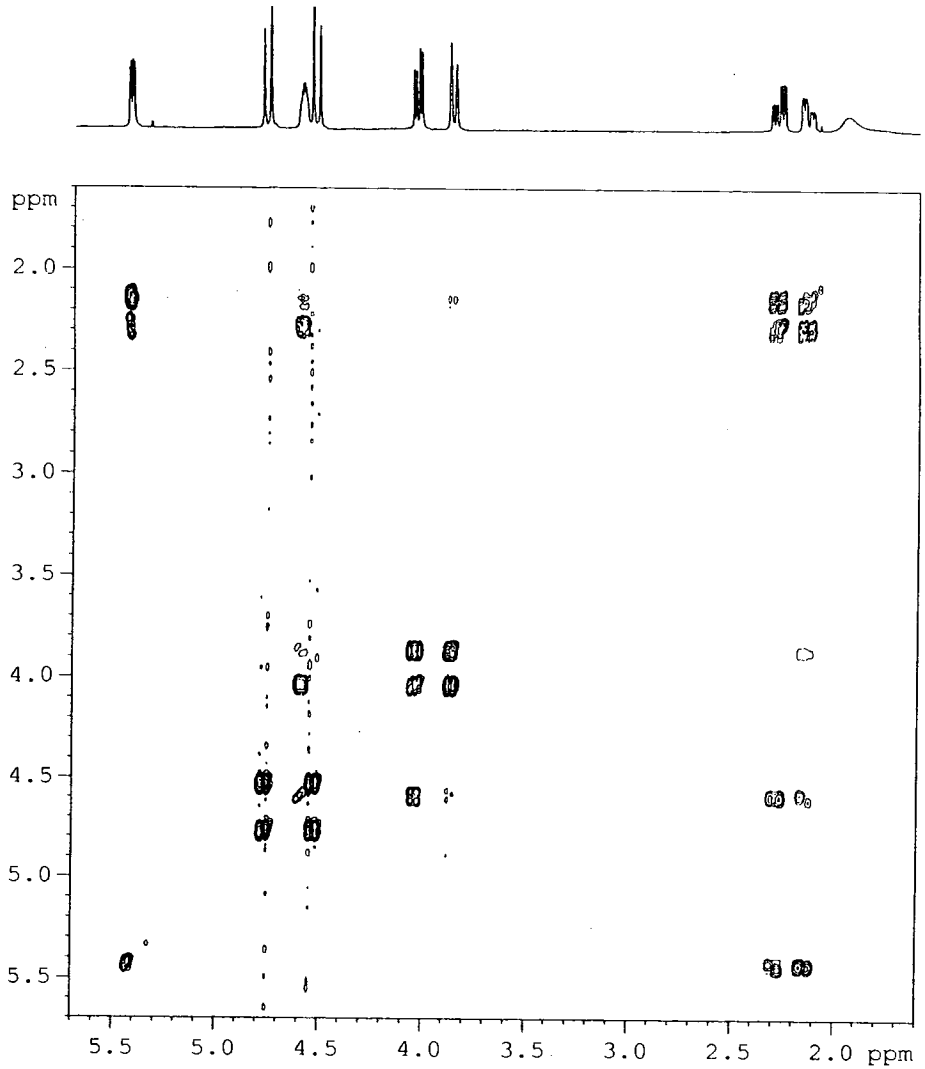
PULPROG         cosygp
TD                512
SOLVENT          CDCl3
NS                   4
DS                   2
SWH           1488.095 Hz
FIDRES        2.906436 Hz
AQ            0.1720820 sec
RG                 256
DW              336.000 usec
DE                6.00 usec
TE                300.0 K
d0           0.00000300 sec
d13          0.00000300 sec
D1           2.00000000 sec
P1                12.85 usec
SFO1       360.1311331 MHz
NUC1                1H
PL1                3.00 dB
P16             1500.00 usec
GPX1               0.00 %
GPY1               0.00 %
GPZ1              10.00 %
GPNAM1          sine.100
D16          0.00015000 sec
P0                12.85 usec
GPX2               0.00 %
GPY2               0.00 %
GPZ2              10.00 %
GPNAM2          sine.100
IN0          0.00067200 sec

F1 - Acquisition parameters
ND0                  1
TD                 128
SFO1        360.1311 MHz
FIDRES     11.625744 Hz
SW             4.132 ppm

F2 - Processing parameters
SI                 512
SF        360.1300000 MHz
WDW              QSINE
SSB                  0
LB                0.00 Hz
GB                   0
PC                1.00

F1 - Processing parameters
SI                 256
MC2                QF
SF        360.1300000 MHz
WDW              QSINE
SSB                  0
LB                0.00 Hz
GB                   0
```

^{1}H-^{1}H COSY of alcohol **121**



SJA 8/70 1H COSY

Current Data Parameters
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PROCNO 1

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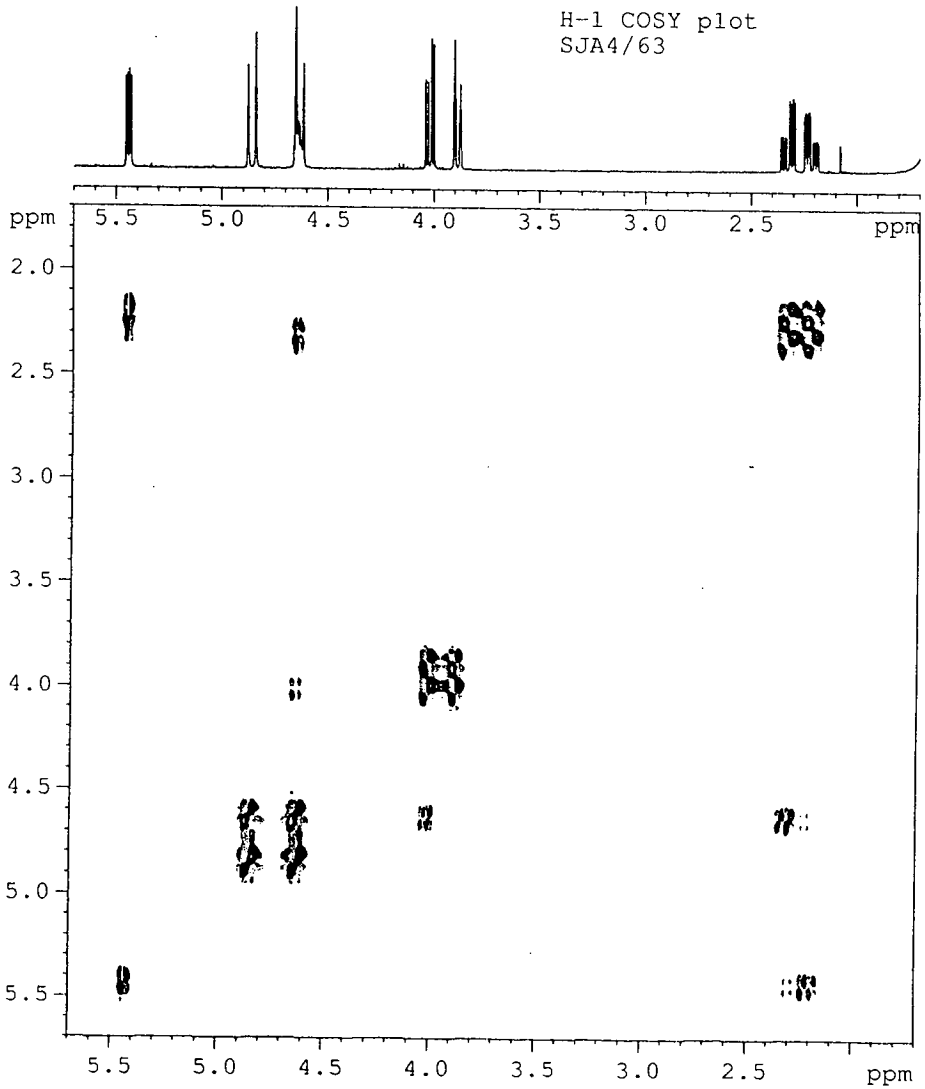
Time 22.32
INSTRUM spect
PROBHD 5mm QNP 1H/11B
PULPROG zg30
TD 32768
SOLVENT CDCl3
NS 32
DS 2
SWH 4310.345 Hz
FIDRES 0.131541 Hz
AQ 3.8011379 sec
RG 322.5
DW 116.000 usec
DE 6.00 usec
TE 300.0 K
D1 0.20000000 sec
P1 13.25 usec
SFO1 360.1319807 MHz
NUC1 1H
PL1 3.00 dB

F2 - Processing parameters
SI 32768
SF 360.1300000 MHz
WDW no
SSB 0
LB 0.00 Hz
GB 0
PC 1.00

^{1}H-^{1}H COSY of alcohol **125**

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Current Data Parameters
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PROCNO                   1

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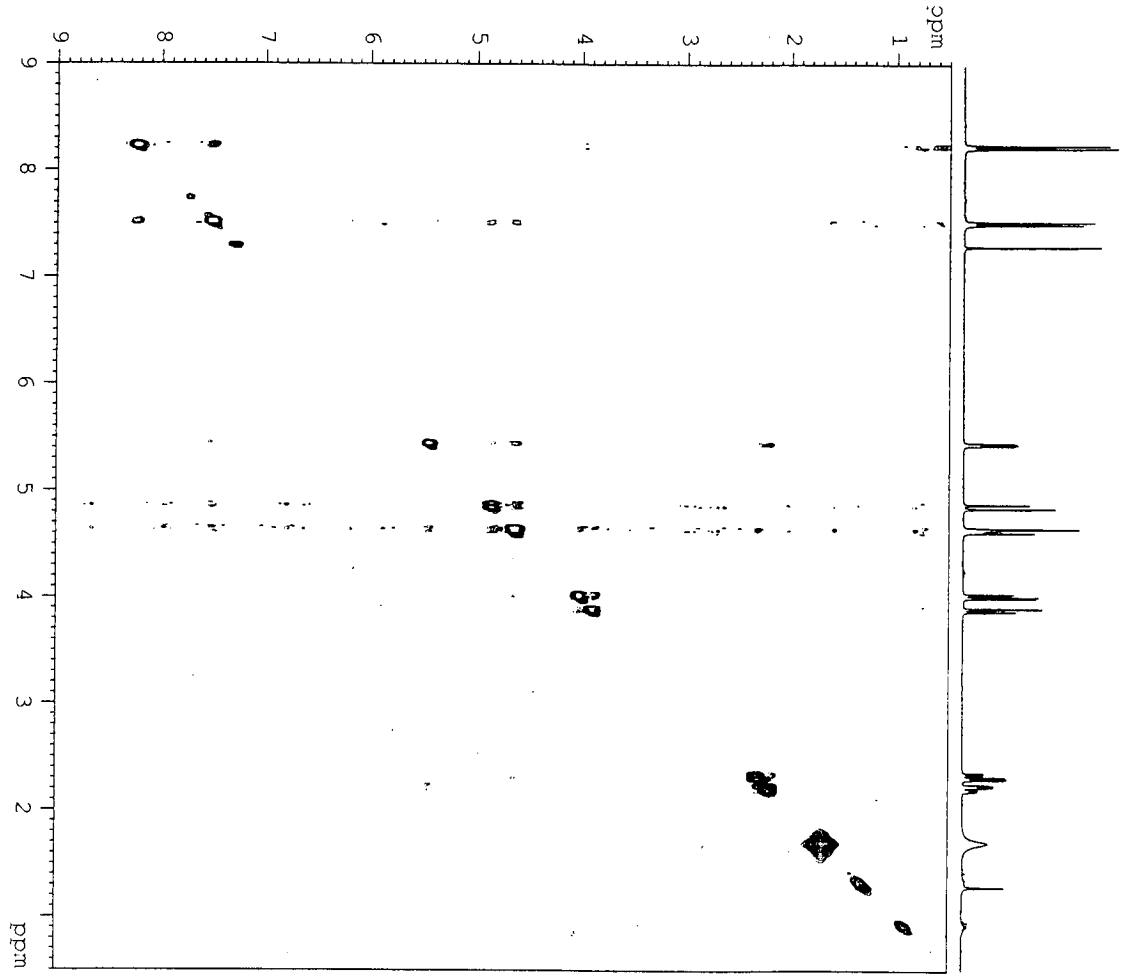
PROBHD     5 mm Multinu
PULPROG          cosygpmftp
TD                     1024
SOLVENT               CDCl3
NS                        4
DS                        4
SWH                2688.172 Hz
FIDRES             2.625168 Hz
AQ                 0.1905140 sec
RG                      100
DW                 186.000 usec
DE                   6.00 usec
TE                  300.0 K
d0                 0.00000300 sec
d13                0.00000300 sec
D16                0.00015000 sec
P16                1500.00 usec
d20                0.00165300 sec
D1                 2.50000000 sec
P1                   12.85 usec
SFO1              360.1317442 MHz
NUC1                     1H
PL1                   3.00 dB
P2                   25.70 usec
GPX1                  0.00 %
GPY1                  0.00 %
GPZ1                 10.00
GPNAM1             sine.100
GPX2                  0.00
GPY2                  0.00 %
GPZ2                 20.00 %
GPNAM2             sine.100
IN0                0.00018600 sec

F1 - Acquisition parameters
ND0                       2
TD                      256
SFO1              360.1317 MHz
FIDRES            10.500672 Hz
SW                 7.464 ppm

F2 - Processing parameters
SI                     1024
SF              360.1300000 MHz
WDW                    QSINE
SSB                       2
LB                     0.00 Hz
GB                        0
PC                     1.00

F1 - Processing parameters
SI                      512
MC2                    TPPI
SF              360.1300000 MHz
WDW                    QSINE
SSB                       2
LB                     0.00 Hz
GB                        0
```

^{1}H-^{1}H COSY of alcohol **133**

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PROCNO                1

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Time                  11.10
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PULPROG               noesytpsi
TD                    1024
SOLVENT               CDCl3
NS                    8
DS                    16
SWH                   3063.726 Hz
FIDRES                2.991920 Hz
AQ                    0.1671668 sec
RG                    225
DW                    163.200 usec
DE                    6.00 usec
TE                    300.0 K
d0                    0.00000300 sec
d11                   0.03000000 sec
L0                    1
L3                    3.00000000
P1                    12.40 usec
SFO1                  360.1317097 MHz
NUC1                  1H
PL1                   3.00 dB
D8                    2.00000000 sec
TD0                   0.00032640 sec

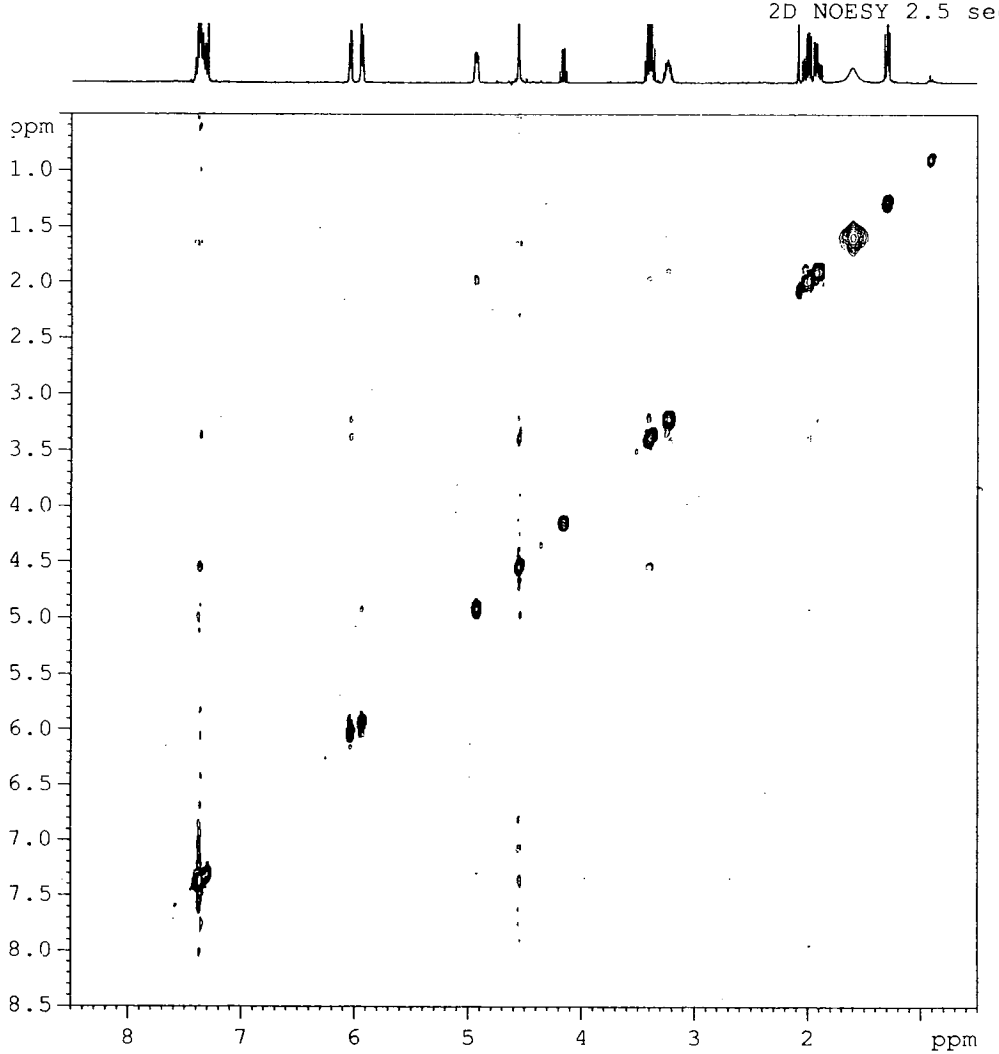
F1 - Acquisition parameters
NUO                   1
TD                    256
SFO1                  360.1317 MHz
FIDRES                11.967678 Hz
SW                    8.407 ppm

F2 - Processing parameters
SI                    1024
SF                    360.1300000 MHz
WDW                   QSINE
SSB                   2
LB                    0.00 Hz
GB                    0
PC                    1.00

F1 - Processing parameters
SI                    512
MC2                   States-TPPI
SF                    360.1300000 MHz
WDW                   QSINE
SSB                   2
LB                    0.00 Hz
GB                    0
```

^{1}H-^{1}H NOESY of alcohol **133**

SJA 11/11
2D NOESY 2.5 sec mixing time




```
Current Data Parameters
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EXPNO                5
PROCNO               1

F2 - Acquisition Parameters
Date_          20000601
Time               17.16
INSTRUM            spect
PROBHD     5 mm Multino
PULPROG        noesyjvst
TD                 1024
SOLVENT            CDCl3
NS                   32
DS                   16
SWH           3238.342 Hz
FIDRES        3.162443 Hz
AQ            0.1581556 sec
RG                  102
DW              154.400 usec
DE                6.00 usec
TE               300.0 K
d0            0.00000309 sec
d11           0.03000000 sec
L0                   1
L3                  45
D1            3.00000000 sec
P1                8.00 usec
SFO1        360.1318007 MHz
NUC1                1H
PL1               3.00 dB
D8            2.50000000 sec
IN0           0.00030880 sec

F1 - Acquisition parameters
ND0                  1
TD                 256
SFO1          360.1318 MHz
FIDRES       12.619774 Hz
SW                8.992 ppm

F2 - Processing parameters
SI                1024
SF          360.1300000 MHz
WDW               QSINE
SSB                  2
LB                0.00 Hz
GB                   0
PC                1.00

F1 - Processing parameters
SI                 512
MC2          States-TPPI
SF          360.1300000 MHz
WDW               QSINE
SSB                  2
LB                0.00 Hz
GB                   0
```

^{1}H-^{1}H NOESY of alcohol **153**